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SYNTHESIS AND EVALUATION OF NATURALLY OCCURING HALOGENATED  
BIPYRROLES IN A RELEVANT MARINE SPECIES, *FUNDULUS HETEROCLITUS*

A Thesis  
Presented for the  
Master of Science  
Degree  
The University of Mississippi

Kimberly Sheree Foster

December 2012





## **ABSTRACT**

Halogenated dimethyl-2-2'-bipyrroles (HDBPs) have recently been discovered in seabird eggs and have been shown to bioaccumulate in trophic organisms. HDBPs are suspected to be biogenic in nature, derived from evidence of marine natural products that are persistent in the environment and widespread in the Pacific and Atlantic Ocean. The HDBPs exhibit chlorinated and brominated substitution patterns that closely resemble anthropogenic pollutants like the polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), and polybrominated diphenyl ethers (PBDEs). Environmental health concerns exist regarding these naturally occurring HDBPs because several of these analogs were shown to induce the cytochrome P4501A (CYP1A) gene through activation of the AhR signaling pathway, similar to other anthropogenic pollutants. They also result in porphyrin accumulation in chicken embryo hepatocytes. Based on these studies, we have focused our efforts on examining the effects of HDBP's to inhibit heme oxygenase (HO), the enzyme responsible for the catalytic oxidation of heme, which may account for the accumulation of porphyrins in hepatocyte models. Within the current study we have synthesized and characterized several natural product halogenated bipyrroles, and examined their effect on heme oxygenase-1, utilizing assays performed on liver lysates obtained from mice treated with the heme oxygenase-1 inducer, cobalt protoporphyrin. The ability of the halogenated bipyrroles to inhibit HO was evaluated by measuring the amount of bilirubin formed from the HO-catalyzed oxidation of hemin. Several of the analogs were

found to be modest inhibitors of HO, and these results suggest that porphyrin accumulation from halogenated bipyrrole exposure may be mediated by the direct inhibition of heme oxygenase-1. We have also focused our efforts on examining the developmental toxicity in a marine relevant species, *Fundulus heteroclitus*. We measured also the induction of the cytochrome P4501A (CYP1A) gene through activation of AhR signaling pathway by HDBPs. *Fundulus* are considered to be the “premier teleost model” in marine biology research, having several functionalized genes capable of tolerating exposure to many environmental contaminants. Exposure of three HDBP’s to *Fundulus* revealed that several of these analogs exhibited significant EROD induction, to negative and positive controls DMSO and PCB126, respectively.

## **DEDICATION**

This work is dedicated to my son Landon C. Foster,  
whose undying love and patience kept me motivated to continue through with my  
graduate studies

## LIST OF ABBREVIATIONS AND SYMBOLS

2,3,6-TBA	2,3,5- Trichlorobenzoate
2,3,7,8- TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
7-ER	7-Ethoxyresorufin
$^{14}\text{C}$	Radiocarbon
$\text{Ac}_2\text{O}$	Acetic anhydride
AhR	Aryl hydrocarbon receptor
ASW	Artificial seawater
ATP	Adenosine triphosphate
BaP	Benzo(a)pyrene
CAS	Chemical Abstract Services
$\text{CH}_3\text{NH}_2\cdot\text{HCl}$	Methylamine hydrochloride
CTCF	Corrected total cell fluorescence
CYP1A	Cytochrome P451A Enzyme
DCM	Dichloromethane
DDE	Dichlorodichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DMF	<i>N,N</i> -Dimethylformamide
DPF	Days post fertilization



E <sup>+</sup>	Electrophile
EPOC	Emerging pollutant of concern
EPA	Environmental Protection Agency
EROD	Ethoxyresorufin- <i>O</i> -deethylase
HAHs	Halogenated aromatic hydrocarbons
HBC	Hexachlorobenzene
HDBPs	Halogenated dimethyl-2-2'-bipyrroles
HO	Heme Oxygenase
HSP-32	Heat shock protein-32
K <sub>ow</sub>	Octanol/water partition coefficient
MeI	Iodomethane
Me-MgI	Methylmagnesium iodide
MeOH	Methanol
MeO-PBDEs	Methoxylated polybrominated diphenyl ethers
MBPs-	1'-methyl-1,2'-bipyrroles
NaH	Sodium hydride
NaOAc	Sodium acetate
NBS	<i>N</i> -Bromosuccinimide
NCS	<i>N</i> -Chlorosuccinimide
PAHs	Polycyclic aromatic hydrocarbons

PBBs	Polybrominated biphenyls
PBDEs	Polybrominated diphenyl ethers
PBTs	Persistent bioaccumulative toxic substances
PCBs	Polychlorinated biphenyls
PCC	Pyridinium chlorochromate
POPs	Persistent organic pollutants
$R_f$	Retention factor
THF	Tetrahydrofuran
TSCA	Toxic Substances Control Act

## ACKNOWLEDGMENTS

I would like to thank Dr. John Rimoldi with much gratitude for his constant guidance, support, and mentorship throughout the completion of my Master's Thesis. His enthusiasm of his work and his energetic approach he uses to conquer daily academics and research has made me realize that once you find something you love to do as a career, work is not actually work anymore. His level of professionalism in the workplace is outstanding and has molded me into a better individual.

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Special thanks go out to my committee members Dr. Marc Slattery and Dr. Kristine Willett for all their input, guidance, and playing a huge part in the Environmental Toxicology Research Program. Thanks go out to Dr. Willett and her research group for the use of their facilities and helping me with the second part of my thesis research. All of you are tremendous individuals, especially Cammi and Meghan who have played a part in helping with my research. I would also like to thank the Environmental Toxicology Research Program for their huge support. Special thanks to the Department of Medicinal Chemistry and their staff for their tremendous support throughout my graduate studies. Much thanks to Dr. Stephen Cutler for helping me as a student outside the department. A special thanks also to Dr. Slattery for his financial support of my side projects that I had the pleasure to work on.

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## I. INTRODUCTION

Humans, wildlife, and plant species are exposed to potentially significant concentrations of environmental contaminants through sources of air, water, sediment, and food. Beginning after World War II, scientists began to realize that specific contaminants persisted in the environment for extended periods of time migrating through various sources of biota (El-Shahawi et al., 2010). Later pollutants like mercury, polychlorinated biphenyls (PCBs), dioxins, and DDT were found to accumulate in fish tissues at concentrations several times higher than in surrounding water, and they had the potential to affect wildlife and human health (Agardy et al., 2008; El-Shahawi et al., 2010). DDT (dichlorodiphenyltrichloroethane), one of the most widely used pesticides during the 1970's, became an effective tool in agriculture with a great promise of eradicating disease carrying insects and the hopes of saving crops. Needless to say, DDT came with the added expense of having an unintended toxicity to many other organisms for which its initial use was not intended. DDT is now restricted in developed countries but is still used in Africa, South, Asia, Central and South America. The largest river in northern Taiwan, Danshui River, receives liquid effluents and atmospheric fallout resulting from industrial and municipal emissions. These emissions consist of multiple forms of POPs (persistent organic pollutants) that are being transported down river causing extensive toxicity to organisms (Hung et al., 2006). The persistent nature of POPs allows them to be accumulated in the tissues of animals being passed

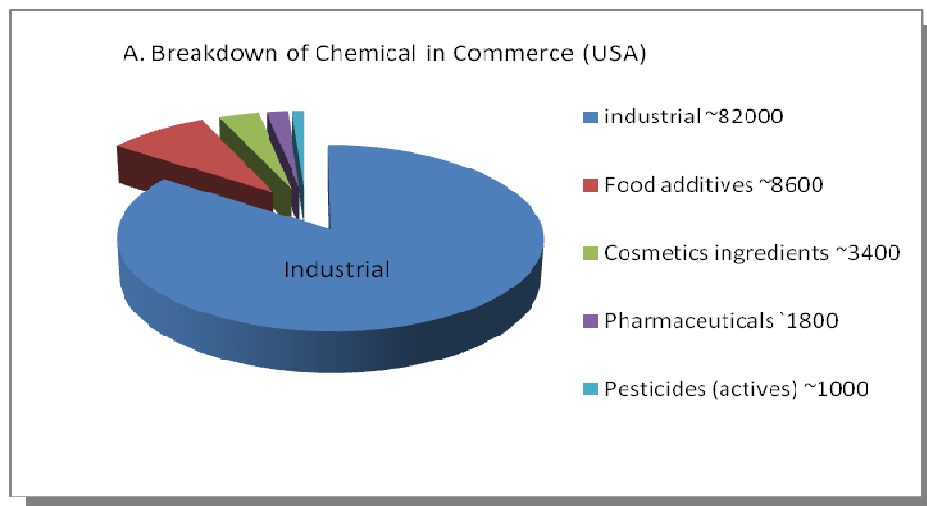
on through the environment at low concentrations migrating to remote places where no initial report of its use has been documented. This has sparked a change in the perception of how nature deals with POPs leading to new scientific discoveries in evaluating its effect on the ecosystem (El-Shahawi et al., 2010).

Ecotoxicology is a discipline within a broader scope of environmental toxicology and the science of ecotoxicology branches into disciplines that include aspects of chemistry and toxicology (Walker et al., 2006). These two areas of knowledge are bridged by the analysis, prediction of the fate and transport of pollutants in the environment, and assessing the adverse effects that a pollutant may pose to an individual organism or an entire ecosystem in a complex environment (Luoma, 1996; Segner, 2007). Scientist Rachel Carson took an superb initiative to warn the public about the use of pesticides, specifically DDT, which she discussed in her striking 1962 book *Silent Spring* (Carson, 1962; Moriarty, 1999). Her awe-inspiring book took the public's knowledge of pesticides to a new level depicting how specific pesticides can affect wildlife. Although not in *Silent Springs*, DDT and its degradation product DDE (dichlorodichloroethylene), were shown to cause an increased risk of mutilation of bird eggs resulting in egg shell lining thinning due to inhibition of calcium-dependent ATPases (adenosine triphosphate) in the egg shell gland. Because DDT and DDE are relatively resistant to degradation, the net result is accumulation of these products in the lipids of higher trophic level birds (Newman and Unger, 2003).

Another extraordinary event that wreaked havoc on the public's view of environmental contamination was the transfer of organic methyl mercury in the marine food web leading to a degenerative neurological disorder called Minamata disease that affected nearly 1000 individuals

with heavy metal poisoning (Harada, 1995; Newman and Unger, 2003). These incidents, amongst many others, changed the public's perception of the common use of chemicals and increased the awareness of the dangerous consequences some pollutants can trigger in the environment.

According to CAS, a division of the American Chemical Society that maintains a database pertaining to chemical information, approximately 15,000 new chemical substances are added to the CAS registry each day (Services). An even more amazing statistic is the number of chemicals in commerce. The U.S Environmental Protection Agency (EPA) Toxic Substance Control Act (TSCA) regulates the screening and testing of new and existing chemicals that enter in commerce periodically for the protection of environmental health (Bowes et al., 1995). Approximately 100,000 chemicals are registered in commerce in the U.S and include pesticides, food additives, cosmetics, industrial chemical, and pharmaceuticals, Figure 1, (Muir and Howard, 2006). With this in mind, the monitoring and screening of new and existing substances is very important for human health and the ecosystem.



**Figure 1**, Total chemicals registered in commerce in USA<sup>a</sup> in US Toxic Substances Control Act (TSCA) inventory over past 30 yrs (*Environ. Sci. Technol.*, **2006**, 40, 7157-7166)

Many environmental programs have been implemented to overcome challenges when dealing with the issues of measuring and monitoring concentrations of chemicals in the environment. Of the chemicals that are represented in industry and commerce, much focus has been aimed towards chemicals that exceed normal background levels in nature and induce biochemical or physiological changes (birth, growth, or mortality rates) in an organism (Walker et al., 2006). Many classical contaminants, such as, DDT and DDE, are already heavily regulated and since have decreased in concentration in the environment (Segner, 2007). Looking ahead, there are always new contaminants emerging for which the toxic mode of action and respective adverse effects has not been accounted. Questions about sources of contamination, distribution, and transport are now surfacing. Within the last decade, halogenated dimethyl-2-2'-bipyrrroles (HDBP's) have been discovered in seabird eggs and bioaccumulating in trophic organisms. These compounds are suspected to be biogenic in nature, derived from evidence of marine

environmental samples that are persistent in the environment and widespread in the Pacific and Atlantic Ocean (Tittlemier et al., 1999). These compounds exhibit chlorinated and brominated substitution patterns that closely resemble anthropogenic persistent organic pollutants (POPs) like the polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), and polybrominated diphenyl ethers (PBDEs). The research efforts described in this thesis will outline the toxicological effects associated with these novel HBDPs through the use of synthetic chemistry and biological assays in a relevant marine species, *Fundulus heteroclitus*.

## 1.1 PROPERTIES OF PERSISTENT ORGANIC POLLUTANTS

**Table 1.** List of Persistent Organic Pollutants

Pesticides	Industrial products	By-products of combustion and industrial processes
Aldrin	Polychlorinated biphenyls (PCBs)	Polycyclic aromatic hydrocarbons (PAHs)
Chlordane	Polybrominated biphenyls (PBBs)	Polychlorinated dibenzo- <i>p</i> -dioxins (PCDDs)
DDT	Polybrominated diphenyl ethers (PBDEs)	Polychlorinated dibenzofurans (PCDFs)
Dieldrin		Polybrominated dibenzo- <i>p</i> -dioxins (PBDDs)
Endrin		Polybrominated dibenzofurans (PBDFs)
Heptachlor		
Hexachlorobenzene (HCB)		
Mirex		
Toxaphene		

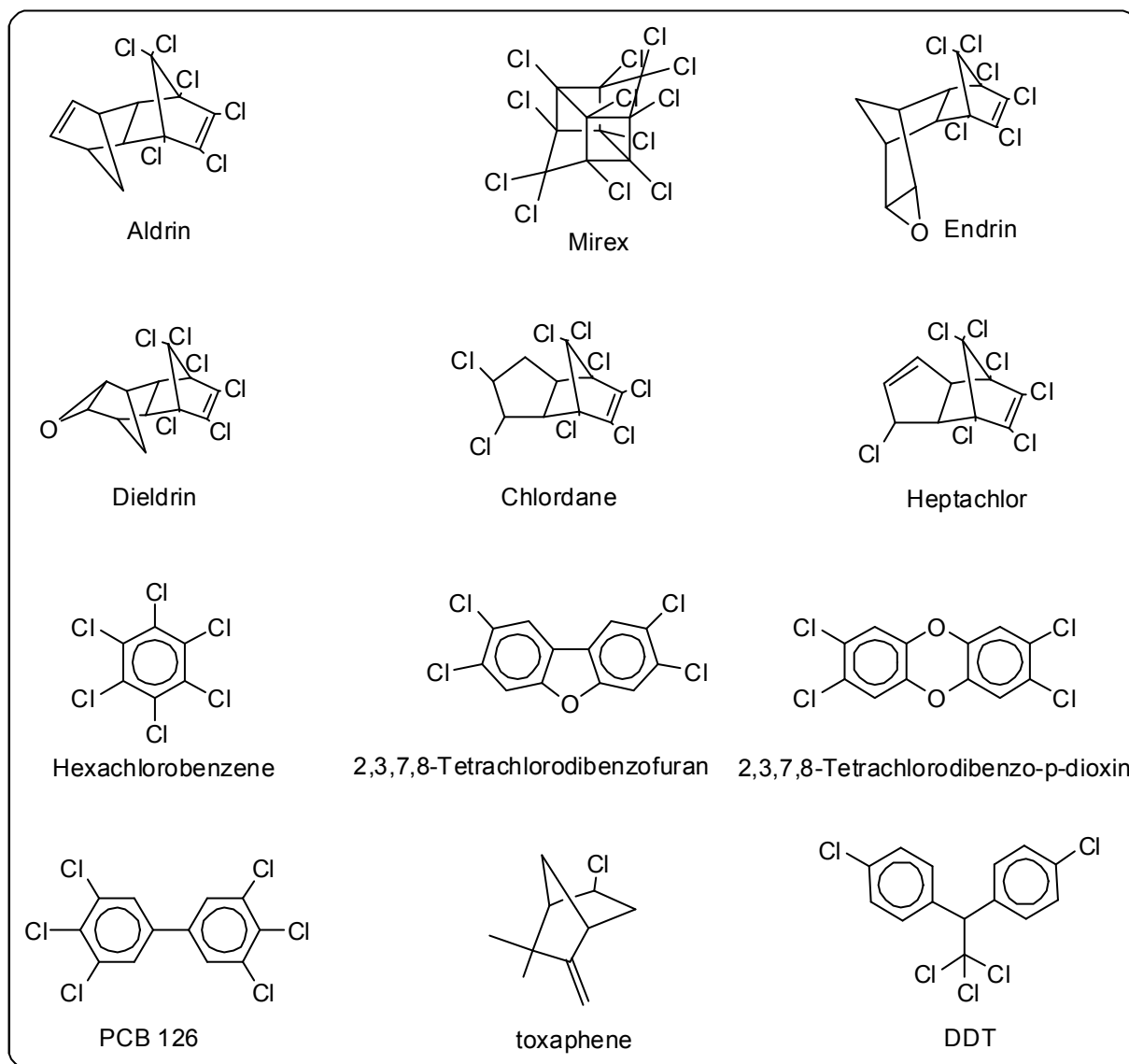
Within the modern era of ecotoxicology, a large body of research has focused on the fate, transport, and toxicity POPs, also referred to as persistent bioaccumulative toxic substances (PBTs) (Table 1). It is these pollutants that are more likely to cause adverse health and environmental effects and subject to long range transport if released into the ecosystem. These

chemicals are notorious for their persistent and bioaccumulative characteristics. POPs exhibit many essential physical and chemical properties that contribute to their unrelenting ability to accumulate in the lipids of higher trophic level organisms. These chemicals have very limited water solubility and very long half-lives in soils, sediments, air, and biota. Although there is no consensus about how long the half-life should be in order to be persistent, persistence depends on an array of other factors such as temperature, incidence of sunlight, and the presence of reactive species (Jones and de Voogt, 1999; Vallack et al., 1998). POPs are highly noted for the semi-volatility, allowing them to partition in the gas phase and be absorbed onto atmospheric particles facilitating re-deposition in the environment and long range transport. Deposition can occur by 1.) adhering to solid or dust particles, soils and sediments, which are then transferred to the surface of ground or water or 2.) by wet deposition and particle absorption by precipitation in the vapor phase, (El-Shahawi et al., 2010). These properties allow POPs to be highly bioavailable with concentrations of several magnitudes of order found in some organisms. These chemicals biomagnify and accumulate in the food chains of trophic organisms at relatively low concentrations (El-Shahawi et al., 2010; Jones and de Voogt, 1999; Ritter et al., 1995; Vallack et al., 1998). Most POPs are halogenated aromatic derivatives, and include PCBs, PPBs, PCDD/Fs, PBDEs, and the organochlorine pesticides. The organochlorines are most relevant because these contaminants are highly resistant to metabolic or photolytic degradation due to chlorine substitution. The carbon-chlorine bond in aromatic structure is able to withstand chemical hydrolysis or photochemical oxidation. As the number of chlorine substitution increases the greater their resistance towards both enzymatic and non-enzymatic degradation. Another major group of POPs are the polycyclic aromatic hydrocarbons (PAHs) which are formed principally



by the incineration of organic compounds (El-Shahawi et al., 2010; Jones and de Voogt, 1999; Ritter et al., 1995).

In an initiative to form a global monitoring program, 92 countries agreed at the 2001 Stockholm Convention to diminish or eliminate 12 original POPs which were coined the name the “dirty dozen”. The dirty dozen were divided (Figure 2) into three classes: pesticides, industrial chemicals, and industrial emissions. The dirty dozen includes aldrin, endrin, chlordane, DDT, dieldrin, heptachlor, mirex, toxaphene, hexachlorobenzene (HCB), which are pesticides, and PCBs, an industrial chemical, and PCDD/Fs, which are by-products of combustion, Figure 2, (2000; 2005; El-Shahawi et al., 2010; Fielder, 2003).



**Figure 2.** Structures of Common Persistent Organic Pollutants

## 1.2 TRANSPORT AND DISTRIBUTION

Pollutant's mode of entry into the environment depends on their pattern of usage, form of application, and means of disposal, especially when dealing with industrial and agricultural waste. Upon release, these chemicals are transported by air and water and are subject to chemical and photochemical biodegradation processes that shed light to how they are eliminated in the

environment. These biodegradation products can exist in many environmental compartments such as sediment, ground and surface water, and wastewater-treatment processes all of which can end up in aquatic environments through run-off or atmospheric deposition. The fate and toxicity of pollutants and their metabolites depend on their ecotoxicological profile and mode of action (Farre et al., 2008). Attributable to the physical and chemical properties POPs demonstrate, they are able to move through the environment to substantial distances, even to remote regions like the Arctic and marine ecosystems where no reported local use of these chemicals has been documented. The long-range atmospheric transport of these chemicals involves a multifarious cycle that involves deposition and revolatilization back into the environment by evaporation where they eventually migrate and accumulate in Polar regions, commonly referred to as “global distillation.” This “global distillation” effect depends on various factors such as temperature, vapor pressure and solubility of the pollutant in water. Molecules in the vapor phase will have a propensity to condense and be carried by northward winds from places of lower latitudes to higher latitudes. The time course and fate of particular chemical depends on its affinity to partition in soil, water, or lipid molecules and contributes to the particular pathway it may take in its journey throughout the environment (Agardy et al., 2008; Rodan, 2002; Vallack et al., 1998).

Given that exposure to environmental pollutants can occur through air, water, or food supplies, the organisms presumably exposed to toxic concentrations of environmental pollutants are predators higher up in the trophic ladder such as marine animals, birds, and larger fish species (Agardy et al., 2008). The miraculous journey of a pollutant can be very complex; however, its bioaccumulative properties can attribute to how these pollutants end up at the top of the trophic chain and elevate to relatively high concentrations through biomagnification. The

food chain transfer can occur in aquatic environments in which pollutants are consumed by filter-feeders and plankton. It was shown that POPs such as DDT, HCB, and PCB are absorbed directly from water (Thomann et al., 1992; Vallack et al., 1998). Biomagnification factor enables POPs to be highly resistant to chemical and metabolic degradation allowing them to be even more concentrated (Vallack et al., 1998). Efforts have been focused on studying the dynamics of transfer in the Great Lakes with an “algae-plankton-fish-fish-birds” pathway as a mode of entry for environmental contaminants in aquatic ecosystems (Hebert et al., 1997; Skoglund et al., 1996). Food chain transfers in terrestrial systems have also been thoroughly studied with the concept of “air-grass-grazing-animal-milk/meat” for human exposure (Jones and de Voogt, 1999; McLachlan, 1995; Welsch-Pausch and McLachlan, 1998). In the midst of the challenges environmental scientists face, studying aquatic ecosystems remains important when looking for early warning signals of new emerging environmental contaminants.

### 1.3 EFFECTS OF POPS IN RELATION TO HUMAN HEALTH

POPs are capable of bioaccumulating in trophic organisms with the ability to travel long distances, making them very persistent and bioavailable in many sources of biota. Due to this nature, increased exposure to these environmental contaminants can cause many adverse health effects among wildlife and humans. Several studies have linked environmental exposure to POPs to numerous adverse health effects such as immunotoxicity (Barnett et al., 1987; De Guise et al., 1995; Martineau et al., 1987), endocrine disruption (Mnif et al., ; Safe, 2000; Toppari et al., 1996; Tyler et al., 1998a; Tyler et al., 1998b), reproductive and developmental effects (Fry, 1995; Gilbertson et al., 1991; Mac and Edsall, 1991; Ouellet et al., 1997), carcinogenesis (Moller et al., 2008; Nagayama et al., 1992; Perera, 1981), and neurotoxicity (Andersen et al., 2000; Eriksson and Fredriksson, 1996). The extent of adverse health effects to which exposure to persistent organic pollutants can cause is beyond the scope of this thesis but several important aspects will be discussed.

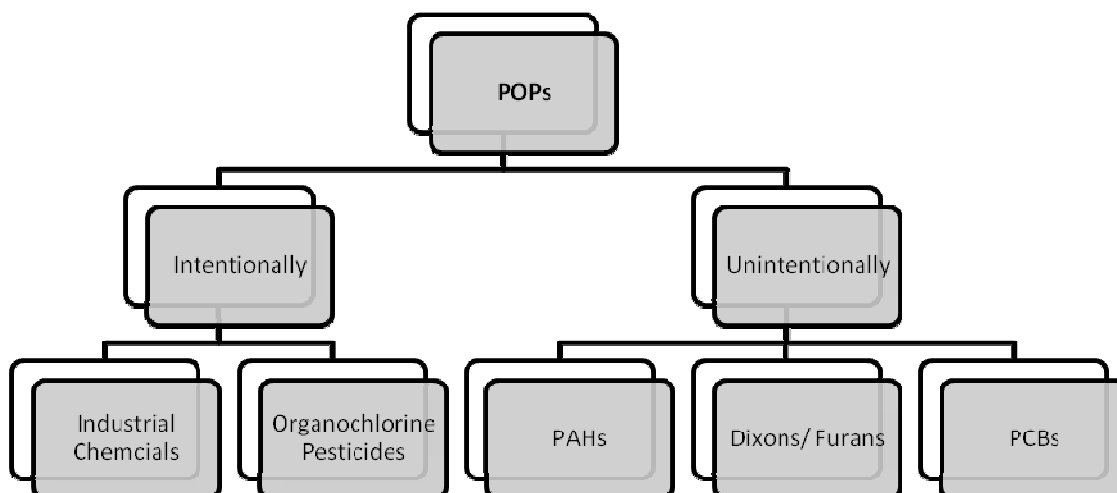
Humans are largely exposed to POPs through dietary intake (Huang et al., 2006). Provided that exposure to environmental pollutants can occur through air, water, or food supply, the organisms presumably exposed to toxic levels of environmental pollutants are those animals that are predators (marine animals, birds, and larger fish species) which is why a great deal of research has been focused towards aquatic ecosystems (Agardy et al., 2008). The amount of seafood consumption is increasing globally. It is estimated that over two billion people globally depend on seafood as key source of protein in their daily diet (1999; Fleming et al., 2006) the most significant documented and distinct evidence of adverse health effects of POPs is in birds and marine animals. Other studies have shown the in-depth effects of reproductive system

deterioration in fish eating birds reported in the Great Lakes and Europe (Bosveld and Berg, 1994; Giesy et al., 1994; Jones and de Voogt, 1999). Nevertheless it is clear that continued exposure to persistent environmental contaminants can result in a gradual deterioration in health, but responses to contaminant exposure is marked as a continuous series of biochemical, physiological, population, and community changes and not just how these responses can be detected in nature (Bayne, 1985; Couillard et al., 1995; Luoma, 1996; Moriarty, 1999).

The most basic effects of pollutants surface at the molecular level where relatively low concentrations of POPs are needed to disrupt critical biochemical functions such as metabolic, hormonal, immune, or genetic processes (Vernberg et al., 1982). Biomarkers can be used to detect changes in cellular processes or biochemical functions and are used as indicators of pollutant exposure (Huggett et al., 1992; Peakall, 1994; Van der Ost et al., 2003). One of most well-known example of a biomarker is the metabolizing enzyme cytochrome P4501A (CYP1A) which is induced by chemicals that activate the aryl hydrocarbon receptor (AhR). This enzyme is a classic biomarker for chemical pollutants like PCBs and dioxins which bind effectively to AhR receptor and induces a series of events that result in the receptor binding to a particular DNA sequence increasing the production of the CYP1A enzyme. 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) is a potent agonist of the AhR receptor and consequently causes toxic effects at minimal doses (Eljarrat and Barcelo, 2003; Luoma, 1996; Segner, 2007). Given that changes at the molecular level are linked to physiological changes in organisms, there are many challenges environmental toxicologists face.

#### 1.4 SOURCES OF PERSISTENT ORGANIC POLLUTANTS

POPs are generally classified as anthropogenic or biogenic in nature. Most POPs are derived from anthropogenic sources and result from either intentional releases, such as pesticide use, or accidental releases, such as byproducts of combustion from the manufacturing certain chemicals or by human activities (Agardy et al., 2008; Breivik et al., 2004; Ritter et al., 1995). Recognizing from what sources POPs originate from provides incentives on reduction measures to eliminate these chemicals in the environment.



**Figure 3.** Classification of POPs (*Talanta*, **2010**, 1587-1597)

Among the intentionally released POPs are pesticides and industrial chemicals. Pesticides include insecticides, herbicides, and fungicides primarily used in the agricultural sector. These chemicals are highly lipophilic and are typically synthetic organic compounds or natural inorganic materials used in pest control management. Many of the organochlorine pesticides used date back to the 1940's. Linkage to neurodegenerative toxicities has been revealed by these

chemicals. Many are banned today in industrialized countries but are prominently used in developing countries. In particular, DDT is still recommended in controlling mosquitoes in developing countries to prevent the outbreak of malaria and other insect vector-borne diseases (Vallack et al., 1998). Although the forbidden use of many organochlorine pesticides has declined, trace concentrations of these chemicals still affect surrounding ecological systems globally (Aspelin, 1994; Chen et al., 2007; El-Shahawi et al., 2010).

PCBs are highly chlorinated structures where at least 2-10 chlorine atoms are attached to a biphenyl ring. The measure of chlorine substitution to the biphenyl ring allows them to be very persistent and to be transported efficiently throughout the environment. PCBs functional group diversity consists of 209 possible congeners (El-Shahawi et al., 2010; Panic and Górecki, 2006; Vallack et al., 1998; Villa et al., 2003). PCBs were first synthesized in the 1920's and were banned in the early 1970's. The extensive use of PCBs in industrial applications are expansive and include, but are not limited to plasticizers, flame retardants, adhesives, lubricants, and dielectric fluids in transformers and capacitors (D'Amato et al., 2002; El-Shahawi et al., 2010; Vallack et al., 1998). The estimated global production of PCBs is in the order of magnitude of 1.2 million tons (Duursma and Carroll, 1996; Vallack et al., 1998). PCB's can be found in the fatty tissues of fish, birds, and other animals which include cow milk and eggs. Humans have also been exposed to PCBs from food sources in which concentrations can be detected in adipose tissue (Loganathan and Kannan, 1994; Vallack et al., 1998). PCBs can also be found in several effluent water systems. Although PCBs were banned in the 1970's, concentrations are detected today which has revealed their persistent exposure to biota.



Unintentionally released POPs are produced accidentally by combustion of organic materials or by the combustion of chlorine containing compounds through emissions in industrial processes. Of these pollutants, much attention has been paid to the dioxin and furan containing compounds (PCD/Fs). Both of these classes of compounds are not commercially produced and exhibit similar structural and chemical toxicity profiles. Of the 75 possible PCDs congeners and 135 PCDFs congeners, only 17 have been shown to exhibit toxicity. From a global perspective, the central sources of release of these compounds are from incineration of municipal, hospital, and hazardous wastes (El-Shahawi et al., 2010; Seys, 1997; Vallack et al., 1998). Limited information has revealed discrepancies between global and regional emission and deposition estimates (Breivik et al., 2004; Duarte-Davidson et al., 1996; Jones and de Voogt, 1999). Polycyclic aromatic hydrocarbons (PAHs) are carbon and hydrogen containing compounds with 2 or more fused benzene rings. They are the combustion by products resulting from the burning of organic materials such as coal, oil, petroleum, or wood mainly from man-made activities. Some PAHs are considered to be probable human carcinogens, 16 are listed on the US EPA's list of priority pollutants. PAHs can also occur naturally from the combustion of forest fires or volcanoes from the synthesis of plants or other microorganisms. They are found in water, soil, and air and able to withstand long range transport over considerable distances as evidence from being measured in marine environments from the north Pacific to the Mediterranean (1995; Aboul-Kassim and Simoneit, 1995; Baek et al., 1991; El-Shahawi et al., 2010; Gagosian et al., 1981; Howsham and Jones, 1998; Sicre et al., 1987; Vallack et al., 1998).

## 1.5 CHALLENGES AHEAD

There are many challenges that lie ahead for environmental scientists. Emerging pollutants of concern (EPOC) are defined as substances that are not yet regulated which the toxic mode of action and adverse effects have not been accounted for and could possibly pose a threat to environmental ecosystems and human health (Farre et al., 2008). There are classes of EPOCs that include, but are not limited to, drugs of abuse, flame retardants, personal-care products, pharmaceuticals, and industrial additives that have been established as potential hazardous environmental contaminants. New EPOC's are not only introduced into commerce, but new ways to produce and distribute them create unique opportunities for sources of pollution and subsequent entry into the environment (Daughton, 2005). Efforts have focused on examining the fate and toxicity of EPOCs in aquatic environments because most emerging anthropogenic pollutants are disposed of through wastewater-treatment processes and end up in aquatic ecosystems.

Early warning and signaling programs have been established to monitor for new EPOCs, many of which cater to marine organisms or animals associated with aquatic ecosystems. Fish-eating birds, such as ospreys, feed on a variety of fish species that are exposed to environmental contaminants that accumulate in the food chains of aquatic systems. Because ospreys are migratory birds, ongoing monitoring studies measuring the concentrations of POPs and other potential toxic pollutants have shed light on the origin of these pollutants. From 1997 to 2004, osprey populations along the lower region of the Columbia River in Oregon have increased as a result of higher rates of reproduction. This fact has been attributed to an apparent decrease in residual pesticide concentrations, in which the species were still exhibiting reproductive failure

in previous years from exposure to the DDT metabolite, DDE. Additionally, other osprey populations located along other sites have potentially toxic residues in their food sources (Agardy et al., 2008; Elliot et al.).

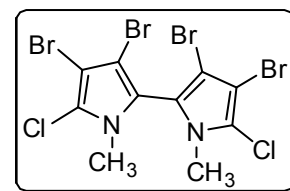
Among other early warning and signaling programs include time-trend studies to monitor the concentration of POPs in marine organisms globally. Time-trend monitoring programs study the rate of decrease of a particular contaminant in an organism of biota over time to get an insight into its persistence. These programs can be used to forecast future impacts or a decline in current toxic impacts (Jones et al., 2002). One of the most well-known time-trend monitoring programs created is the International Mussel Watch Program, which examines the levels of POPs in bivalves around the globe annually. Bivalves are sessile organisms which are able to tolerate and adapt to broad range of contaminants in vast conditions. They are perfect surveillance organisms to monitor the current state of pollution in coastal marine ecosystems (Gevao et al., 2009; Goldberg et al., 1978).

In an on-going initiative to monitor for new EPOCs, unusual natural halogenated compounds were discovered recently in seabird eggs. The first compound is a mixed halogenated organic compound identified as tetrabromo-dichloro-bis(N-methyl)-2,2'-bipyrrole (DBP-Br<sub>4</sub>Cl<sub>2</sub>), while the second is a heptachlorinated 1,2'-bipyrrole derivative (Tittlemier et al., 1999; Vetter et al., 2000; Vetter et al., 1999). These compounds appeared to be accumulating in trophic organisms such as birds, fish, and mammals native to the Pacific and Atlantic Ocean. What is so intriguing about this class of compounds is that they exhibit the characteristics of many anthropogenic POPs such as the polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), and polybrominated diphenyl ethers (PBDEs). The naturally occurring halogenated

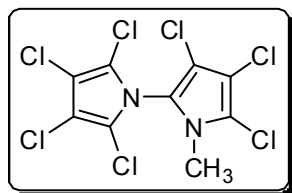
dimethyl-2-2'-bipyrroles (HDBPs) has led to many speculations as to their etiology and since previous known sources of these compounds were considered to be by-products of incineration. Given that these compounds are strikingly similar to other anthropogenic POPs, environmental health concerns began to surface as to what possible adverse health effects these compounds would pose to the environment. The next section will discuss the history behind the discovery, identification, and some relevant health studies regarding these novel natural HDBPs.

## II. HISTORY OF MARINE HALOGENATED PYRROLES AND A REVIEW OF THE LITERATURE

Organohalogen compounds are historically known to be of anthropogenic nature being byproducts of incineration from industrial processes. Nearly 3200 known naturally occurring are produced largely by plants, sponges, tunicates, bacteria, and fungi (W. Gribble, 1999). A little over a decade ago, novel natural halogenated dimethyl bipyrroles (HDBPs) were first identified and isolated in seabird eggs and bald eagle liver samples from the Pacific and Atlantic Ocean in concentrations ranging from 1.8 to 140 ng/g wet weight. At the time of isolation, this natural product was identified as a compound with a molecular formula of  $C_{10}H_6Br_4Cl_2$  (Tittlemier et al., 1999). The structure of the halogenated natural product upon isolation was uncertain. The structure of the compound was later confirmed



**Figure 4.** Structure of DBP- $Br_4Cl_2$



**Figure 5.** Structure of Q1

as 5,5'-dichloro-1,1'-dimethyl-3,3',4,4'- tetrabromo-2,2'-bipyrrole (DBP- $Br_4Cl_2$ ) by Gribble *et al.* by synthesis and comparison with the isolated natural product, Figure 4, (Gribble et al., 1999). Another halogenated bipyrrole derivative was also found in several environmental and biological samples and identified as heptachloro-1'-methyl-1,2'-bipyrrole (Q1),

Figure 5, constitute another family of halogenated natural products called the methylated

bipyrroles (MBPs) (Vetter et al., 1999). Q1 sparked more interest in the halogenated bipyrrole class of compounds because of the exhaustive chlorination in its structure and its occurrence in the environment. The intriguing aspect of the discovery of these halogenated bipyrrole derivatives is that they were suspected to be bioaccumulating in trophic organisms based on isolation from samples such as marine mammals, air, food, and even human milk some of which can be found in Table 2 (Vetter, 2006). These halogenated derivatives of the bipyrrole class have sparked many thought provoking investigations because there is no industrial synthesis of either compound, yet they are distributed throughout the environment. In addition to their occurrence, mixed halogenated compounds are rarely seen in industrial chemicals with the exception of those which are formed during incineration processes (Tittlemier et al., 2002a). Possible point sources were considered, including speculation that they may be unknown metabolites of degradation or byproducts of incineration from industrial processes (Reddy et al., 2004). HDBPs resemble structural similarity to the more common anthropogenic halogenated POPs. There has been speculation that these compounds could have some of the same toxicological characteristics as PCBs or PBDEs. The impact of how HDBPs affect the environment and its toxicity still remain uncertain with scarce toxicological profiles.

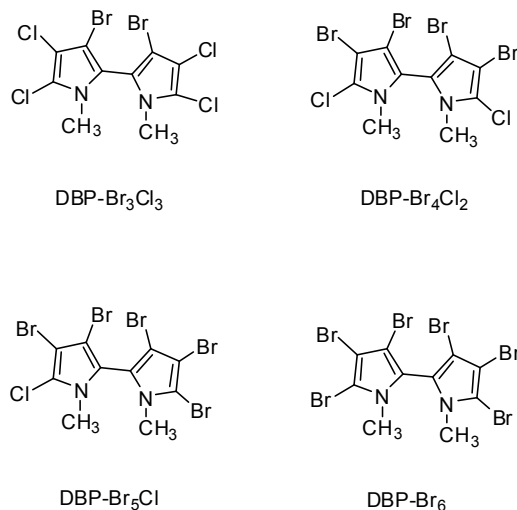
**Table 2.** Concentrations of HDBPs (ng/g lipids) in the marine environment and food (Adapted from *Rev Environ Contam Toxicol*, **2006**, 188, 1-57)

Species	Location	N	sumHDBPs	PCB153	Source
Beluga	North America Svalbard		14-18	540-8,000	Tittlemier et al. (2002b)
Dall's porpoise	NW North Pacific	5	2,540	1,240	Tittlemier et al. (2002b)
Hector's dolphin	New Zealand	5	48	76	Tittlemier et al. (2002b)
California sea lion	California	5	93-9,800	910-90,800	Tittlemier et al. (2002b)
Harbour seals	Various locations	69	0.02-526	65-282,000	Tittlemier et al. (2002b)
Bottlenose dolphin	Australia	4	250-4,150	230-8,800	Vetter et al. (2001a)
Green turtle	Australia	1	26	70	Vetter et al. (2001a)
Marine fish	Canada	62	<0.6-1,100		Tittlemier (2004)
Freshwater fish	Canada	39	<0.6-220		Tittlemier (2004)
Canned fish	Canada	86	25-6,600		Tittlemier (2004)
Shrimp	Canada	33	<0.6-220		Tittlemier (2004)
Sediment	Canadian Arctic	2	-0.03		Tittlemier (2002a)
Arctic Cod	Canadian Arctic	5	1.1		Tittlemier (2002a)
Black guillemot	Canadian Arctic	6	9.5		Tittlemier (2002a)
Seal	Canadian Arctic	10	~0.1		Tittlemier (2002a)
Seabird eggs offshore	Pacific, Canada	19	32-140	~70-100	Tittlemier (1999)

Initial detection of DBP-Br<sub>4</sub>Cl<sub>2</sub> began in 1988 during a screening of seabirds from the Atlantic and Pacific coast in an effort to identify contaminants using low--resolution electron impact ionization mass spectra (EI-MS), (Elliot et al., 1992; Tittlemier et al., 1999). The compound identified was unknown but the molecular formula was determined through high-

resolution mass spectra on contaminated bald eagle liver samples. DBP-Br<sub>4</sub>Cl<sub>2</sub> is the most abundant congener found at ppm concentrations in marine environmental samples, but the structures of other HDBPs congeners found in the environment were later characterized and determined by isotope exchange mass spectrometry, Figure 6, (Tittlemier et al., 2002b; Tittlemier et al., 2002c). After its initial detection and structure confirmation, these compounds showed evidence of being widely distributed in the environment and exhibiting bioaccumulative and persistent properties in a variety of marine samples. A study reported the presence of HDBP congeners in environmental samples of an Arctic marine food web using stable nitrogen isotope analysis (Tittlemier et al., 2002c). The presence of these compounds in zooplankton, fish, seabirds, and seals indicate the widespread nature of their occurrence and moreover, their corridor of distribution stretching from the Pacific, Atlantic, to the Arctic Ocean. This study also showed that the four HDBPs biomagnify in the environment due to an apparent increase in concentration with trophic level in the marine food web examined, with the exception of ring seals, which showed an ability to metabolize HDBPs (Tittlemier et al., 2002c). Marine mammals are good indicators of persistence of particular organohalogen compounds because they accumulate in the lipids at relatively high concentrations. Other studies reported the distribution of HDBPs in North American marine mammal blubber from ringed seals to a species in the cetacean family, Dall's porpoise, with total HDBP concentrations ranging from 0.4 ng/g to 2,540 ng/g lipid weight and detection in whales and dolphins were also seen on the Japanese market (Haraguchi et al., 2006; Tittlemier et al., 2002a).

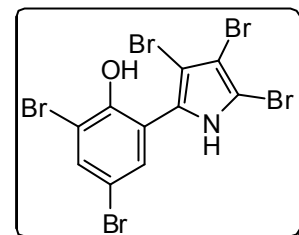




**Figure 6.** Structures of halogenated dimethyl bipyrroles (HDBPs) found in the environment

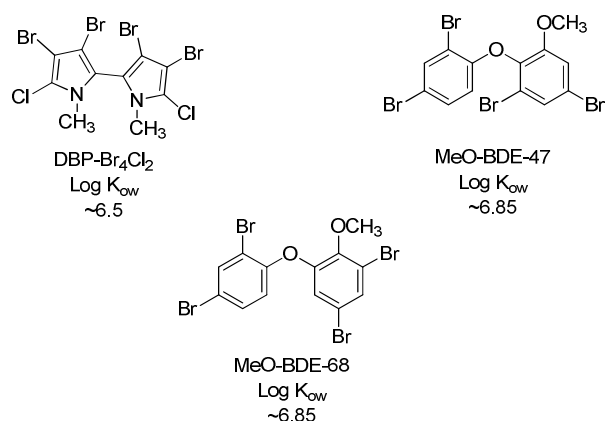
## 2.1 STRUCTURAL AND PHYSICAL PROPERTIES OF HDBPs

HDBPs are halogenated compounds fully substituted with bromine or chlorine atoms with a common 1,1'-dimethyl-2,2'-bipyrrole spine. HDBPs show structural similarity to a known marine natural bacterial product, hexabromo-2,2'-bipyrrole, Figure 7, (Andersen et al., 1974). The X-ray crystal structures of three HDBPs were first reported by Blank et al who noted the expansion of symmetry across the C2-C2' produces the bipyrrole moiety. The pyrrole rings are essentially planar with some bending of halogenated atoms out of the ring plane (Blank et al., 2002). These compounds also revealed structurally similar characteristics to other polyhalogenated biphenyls, but have significantly smaller dihedral bond angles between the ring planes (Blank et al., 2002). Common organohalogen compounds such as the PCBs and PBDEs are exclusively chlorinated and usually



**Figure 7.** Structure of hexabromo-2,2'-bipyrrole

are of anthropogenic nature while many mixed halogenated compounds tend to be byproducts of incineration. One criterion for compounds being lipophilic is compounds with a log  $K_{ow}$  greater than 5 (Vetter, 2006). These compounds show comparable log  $K_{ow}$  (6.5-6.7) values to other penta- and hexachloro biphenyls such as the methoxylated polybrominated diphenyl ethers (MeO-PBDEs) with the structures shown in Figure 8 (Teuten et al., 2005; Tittlemier et al., 2004). The high  $K_{ow}$  values of these compounds suggest that HDBPs may perhaps be transferred through the food webs of aquatic systems and may have the potential to biomagnify up trophic level organisms (Tittlemier et al., 2002c). Trophic transfer is the major exposure pathway for higher level aquatic organisms at the top of the food web (Thomann et al., 1992). Vapor pressures of HDBPs are very low with water solubility ranging from  $1 \times 10^{-5}$ -  $3 \times 10^{-5}$  g/l and a Henry law constant with an estimation of 0.005 Pa m<sup>3</sup>/mol (Tittlemier et al., 2002a; Tittlemier et al., 2004). As expected, vapor pressures decrease with an increase in the number of bromine substituents. The physicochemical parameters of HDBPs are comparable with the properties of PCBs and PBDEs (Mackay et al., 1999). These compounds are pervasive and move through the environment as the more common PCBs, but the extent to how these compounds are distributed may differ and the adverse effects of these compounds to other POPs is still lacking.



**Figure 8.** Log  $K_{ow}$  for DBP-Br<sub>4</sub>Cl<sub>2</sub> and MeOH-PBDEs

## 2.2 CONSIDERATIONS ABOUT DISTRIBUTION, TRANSPORT, AND SOURCE OF ORIGIN

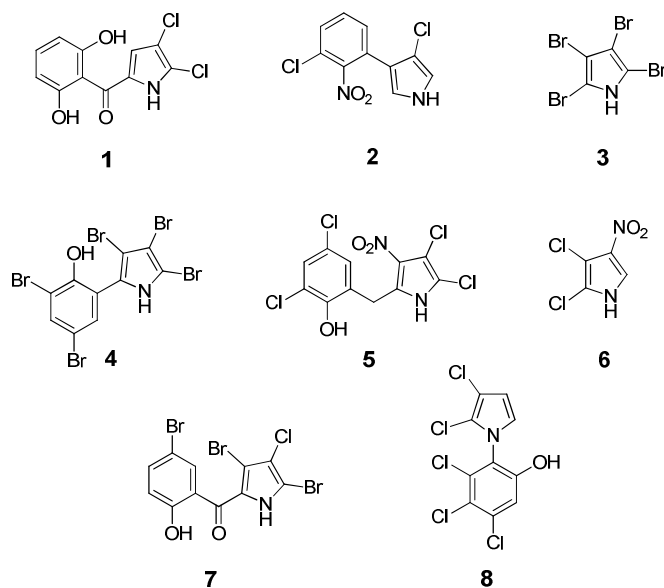
It is clear that HDBPs are more widespread in the environment than previously believed, but there is a difference in the global distribution of these compounds compared to the more widely distributed PCB congeners such as PCB153. It was noted that HDBPs are more frequently detected in Pacific Ocean samples, followed by Atlantic Ocean samples, and an absence in freshwater environments such as the Great Lakes (Tittlemier et al., 1999). This is in stark contrast to the distribution of PCBs, which have highest detection of contaminants in Atlantic Ocean samples, followed by the Northwest Pacific Ocean, Arctic Ocean, and Antarctic Ocean (Muir et al., 1988). The physical properties of both classes of organohalogenes show that there are similarities between groups and suggest that HDBPs may move throughout the environment in the same capacity as the PCBs. Variation in the extent of distribution between these two classes of compounds may be attributable to differences in sources. HDBPs are formed mostly in the Pacific Ocean and the sources of PCBs are distinctly of terrestrial environments

and distributed throughout industrial urban areas (Tittlemier et al., 2002a).

There has been much speculation about the transport mechanisms exhibited by HDBPs to provide explanation to how these compounds are found in the northern Pacific Ocean and the source from which they originate. The two possible modes of transport regulating the distribution of these compounds in marine environments are atmospheric and oceanic current transport. Evidence that may rule out atmospheric transport as the major mode of transport pertains to the fact that these compounds were not found in any Great Lakes seabird samples in a previous study (Tittlemier et al., 1999). Additionally, the HDBP's exhibit very low Henry's law constants similar to the octa- to decachlorinated PCB congeners which are not likely to exhibit extensive atmospheric transport (Mackay et al., 1999). The Henry's law constant describes the ability of a substance to partition in air or water. Compounds with high Henry's law constants will more likely volatilize from the aqueous phase to air while those with low Henry's law constants will tend to remain in its aqueous phase (Tittlemier et al., 2002a). Contrary to this evidence, HDBPs were found at low concentrations in a freshwater environment in Baikal seals in Lake Baikal, Russia. The minimal concentrations in this freshwater environment and its noticeable absence in the Great Lakes may be due to average directional surface wind flows in which flows vary in directions at different times of the year (Hasse and Dobson, 1986; Tittlemier et al., 2002a). Despite the notion that atmospheric transport is not a major mode of transport, oceanic transport is most likely responsible for the global distribution of these compounds from the Pacific, Atlantic, and Arctic regions.

Most organohalogen compounds based on the classic pollutants are of anthropogenic nature from pesticides, industrial uses, or byproducts of incineration. The unusual feature of

HDBPs is that there is no record of industrial synthesis or manufacture of these compounds, though they do exhibit structural similarity to the marine natural bacterial hexabromo-2,2'-bipyrrole and may point towards a biogenic source (Andersen et al., 1974). A number of halogenated pyrroles have been detected in nature since the isolation of pyoluteorin (**1**) from an antibiotic from *Pseudomonas aeruginosa* in 1958 and pyrrolnitrin (**2**) from *Pseudomonas pyrrocinia* in 1965 shown in Figure 9 (Imanaka et al., 1965; Takeda, 1958).



**Figure 9.** Structures of halogenated pyrroles found in nature (**1**) pyoluteorin, (**2**) pyrrolnitrin, (**3**) polybrominated pyrrole, (**4**) hexabromo-2,2'-bipyrrole, (**5**) pyrrolomycin B, (**6**) pyrrolomycin A, (**7**) pyrrolomycin F, (**8**) neopyrrolomycin (*J. Nat. Prod.*, **1992**, 55, 10, 1353-1395)

For example, the marine bacterium *Chomabacterium* species produces the polybrominated pyrrole (**3**) and hexabromo-2,2'-bipyrrole (**4**) (Anderson et al., 1974). The pyrrolomycins exhibit physically powerful antibiotic activity. The soil microbe *Streptomyces* species generates pyrrolomycin B (**5**) and *Actinomyces* species produces pyrrolomycin A (**6**) (Kaneda et al., 1981; Koyama et al., 1981). Pyrrolomycin F is synthesized by the microbe

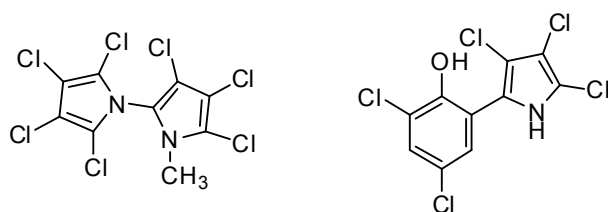
*Actinosporangium vitaminophilum* (7) and neopyrrolomycin (8) is produced by the soil microbe *Streptomyces* species (Ezaki et al., 1983; Koyama et al., 1983). Several other brominated pyrroles are produced by sponges. Several of these pyrrole-based molecules of natural origin have served as pivotal sources of natural product drug discovery in medicinal chemistry due to their various biological and strong antibiotic activities (Bergman and Janosik, 2011; Gribble, 1992).

The distribution analysis of HDBPs indicates that they are 1.) prevalent and formed in marine ecosystems or 2.) subject to long range transport from point sources or regions of formation. These scenarios suggest that the source is either a common organism with a wide occurrence, or the source is formed by an organism with a limited range in the ecosystem (Tittlemier et al., 2002c). Both possibilities cannot solely eliminate a biogenic source, but there may be some indirect evidence for the degree of a natural source in radiocarbon ( $^{14}\text{C}$ ) content. Radiocarbon ( $^{14}\text{C}$ ) content analysis is an excellent technique for drawing conclusions about the origin of organohalogenes. All natural products are inherently prelabeled with some  $^{14}\text{C}$  while synthetic products derived from petroleum should not contain any measurable  $^{14}\text{C}$  with the exception of toxaphene due to its synthesis from a non-petrochemical source. Radiocarbon is produced in the atmosphere and is quickly oxidized to  $^{14}\text{CO}_2$ . Due to the uptake of  $^{14}\text{CO}_2$  by plants for photosynthesis, plants reflect contemporary levels of  $^{14}\text{C}$ . Levels of  $^{14}\text{C}$  then decrease through radioactive decay with a half-life of 5730 years which is why synthetic petroleum products do not contain any measurable  $^{14}\text{C}$  content due to their formation over  $> 50,000$  years (Reddy et al., 2002). DBP- $\text{Br}_4\text{Cl}_2$  was isolated from a marine animal extracts and the  $^{14}\text{C}$  content was determined using radiocarbon analysis by Reddy et al. (2004). Reddy et al. (2004)

determined the  $^{14}\text{C}$  content of DBP- $\text{Br}_4\text{Cl}_2$  to be in order of 5000 years. This value is significantly more depleted than expected and three scenarios were given to explain the drastic depletion of  $^{14}\text{C}$  content. First, there may be a combination of anthropogenic and natural sources in which the  $^{14}\text{C}$  value reflects both equivalent contributions from each source. The second scenario could be utilization of carbon that is aged during natural biosynthesis by an organism. Lastly, the  $^{14}\text{C}$  value may reflect the age of DBP- $\text{Br}_4\text{Cl}_2$  in primitive years since biosynthesis. Although it is unclear to which of these explanations the depletion of  $^{14}\text{C}$  for DBP- $\text{Br}_4\text{Cl}_2$  may possibly refer to, it can be used as proof for a source of natural origin because the  $^{14}\text{C}$  value was measurable. Other evidence that may exclusively point HDBPs towards a natural source is their widespread in marine environments, high accumulation the blubber of marine animals, absence in freshwater environments, and variation in distribution patterns compared to the classical anthropogenic contaminants (Vetter, 2006).

### 2.3 OTHER PROPOSED NATURAL HALOGENATED PYRROLES

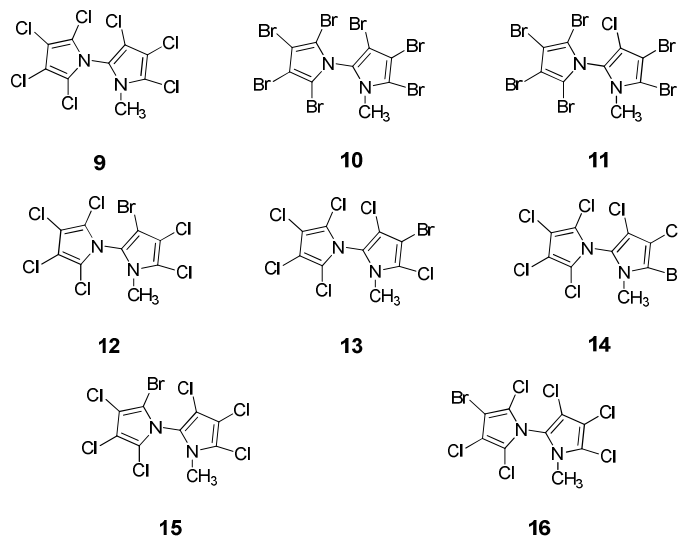
Another unknown halogenated contaminant was detected in marine mammal samples from Africa and Antarctica and was given the name of “Q1” (Vetter, 2000). The molecular formula of Q1 was determined as  $\text{C}_9\text{H}_3\text{Cl}_7\text{N}_2$  using high resolution mass spectrometry (Vetter et al., 1999). The structure was confirmed to be 2,3,3',4,4',5,5'-heptachloro-1'-methyl-1,2'-bipyrrole, Figure 5, (Wu et al., 2002).



**Figure 10.** Structure of Q1 and pentachloro-2-pyrrolyl-2-phenol

The structure revealed two nitrogen-carbon linked pyrrole rings substituted with halogens with a single N-methyl group. The most attractive feature about Q1 is its high degree of halogenation, comprising fully substituted chlorinated pyrrole rings which is very rare to be naturally occurring in nature (Teuten et al., 2006a). Though structural comparison of this compound was made against another halogenated natural product, pentachloro-2-pyrrolyl-2-phenol, isolated from a terrestrial bacterium *Actinoplanes*, Figure 10, Q1 was detected in samples dating back to the 1980's which remained unidentified until the last decade (Cavalleri et al., 1978; Vetter, 2006). This halogenated bipyrrole has been identified in several biological samples including human breast milk from various regions across the globe (Vetter et al., 2000; Vetter et al., 2003). Subsequent detection of Q1 led to the identification of other 1'-methyl-1,2'-bipyrrole (MBPs) congeners which were detected in 2006 and thereafter using high resolution mass spectrometry, Figure 11 (Teuten et al., 2006a; Teuten et al., 2006b; Vetter et al., 2007).





**Figure 11.** Structures of MPB Congeners (**9**) Q1, (**10**) MBP-Br<sub>7</sub> (**11**) MBP-Br<sub>6</sub>Cl (**12**) 3'-Bromo-2,3,4,4',5,5'-hexachloro-1'-methyl-1,2'-bipyrrole (Br-MBP-78), (**13**) 4'-Bromo-2,3,3',4,5,5'-hexachloro-1'-methyl-1,2'-bipyrrole (Br-MBP-76), (**14**) 5'-Bromo-2,3,3',4,4',5,5'-hexachloro-1'-methyl-1,2'-bipyrrole (Br-MBP-74). (**15**) 2-Bromo-3,3',4,4',5,5'-hexachloro-1'-methyl-1,2'-bipyrrole (Br-MBP-75), (**16**) 3'-Bromo-2,3',4,4',5,5'-hexachloro-1'-methyl-1,2'-bipyrrole (Br-MBP-77)

This mono-methylated bipyrrole moiety may be related to the HDBPs that were previously described, because there is no record of its industrial use and the natural producer of this compound remains unknown. Compared to HDBPs, Q1 is more prevalent in the Antarctic region of the Southern Hemisphere. Brominated MBP congeners are more abundant in samples isolated from the Atlantic region (Pangallo et al., 2008; Vetter, 2006; Vetter et al., 2007). Similar to DMBPs, MBP concentrations were lower in ringed seals, and had lower blubber concentrations in harbor seals (Pangallo and Reddy, 2009; Vetter et al., 2003). The log  $K_{ow}$  for Q1 exceeds 5, but is somewhat lower than the HDBPs (Hackenberg et al., 2003; Vetter, 2006). MBPs are presumed to be from a natural source, but just like HDBPs additional studies on marine bacterial cultures may provide the original source from which these compounds are

derived (Reddy et al., 2004).

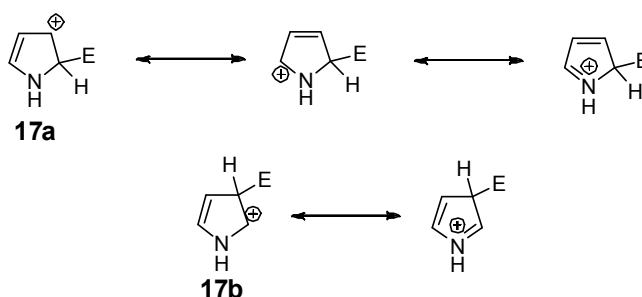
## 2.4 FUTURE NEEDS

HDBPs as well as MBPs such as Q1 are globally distributed. Although these compounds are produced in specific geographical locations, the difference in their geographical distribution may reflect the divergence in sources from which these compounds are derived. These compounds reveal structural similarity to other natural marine bacterial products which may give insight to a confirmed natural source. If these compounds are truly of natural origin, then there may be a possibility that these compounds were present in the environment for a much longer period of time than PCBs. For this reason these compounds may shed light on the long-term fate of other halogenated organic compounds with similar physical properties (Reddy et al., 2004; Teuten et al., 2006a). Furthermore the identification of these compounds in various biota has revealed some environmental health concerns about these halogenated compounds. With the detection of Q1 in human breast milk and HDBPs in whale and dolphin products marketed for human consumption, it is clear that these compounds may pose an exposure or health risk for individuals who consume marine organisms, or wildlife (Haraguchi et al., 2006; Vetter et al., 2000). The toxicological profiles of these compounds are not well known; however, bioactivity studies of these compounds revealed that HDBPs congeners and Q1 bind with the aryl hydrocarbon receptor (AhR) and induce cytochrome P450 activity, which will be discussed in a subsequent chapter (Tittlemier et al., 2003b; Vetter et al., 2004). Lack of commercially available reference standards makes it difficult to establish structure- toxicity relationship analysis of these compounds. This stresses the need for implementing a synthetic chemistry strategy for these

compounds in order to pursue toxicological studies for HDBPs. The next section will outline our synthetic approaches for the halogenated dimethyl bipyrrroles and related compounds. We strategically synthesized two HDBP congeners (DBP-Br<sub>6</sub>, DBP-Br<sub>4</sub>Cl<sub>2</sub>) that are found in nature and a hexahalogenated HDBP derivative (DBP-Cl<sub>6</sub>) as a synthetic standard because its similarity in structure to the exhaustive chlorinated MBP congener Q1.

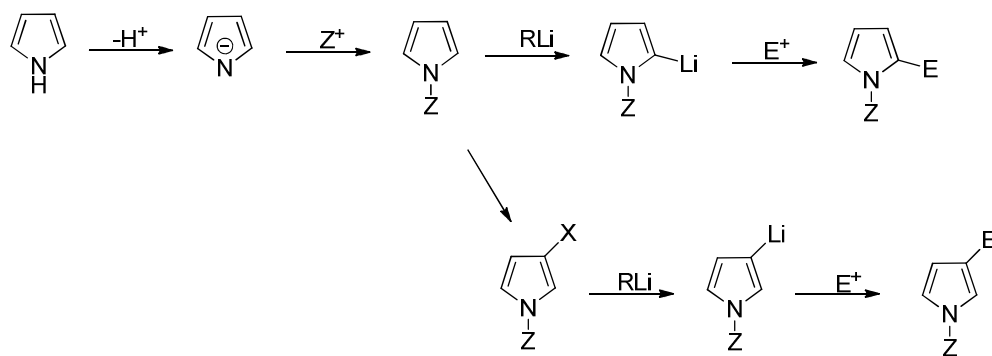
### III. SYNTHETIC APPROACHES OF HALOGENATED DIMETHYL BIPYRROLES AND RELATED COMPOUNDS

In 1834, Runge's keen curiosity led him to observe the presence of a compound obtained from a fraction of coal tar. Runge witnessed the red coloration of a wood splinter dampened with mineral acid caused by the collected fraction. The substance was named pyrrole and a pure sample of this substance was isolated by Anderson by distillation of ivory oil; the structure of the compound was established several years later (Anderson, 1857; Runge, 1834). Ever since the first observation of pyrrole, its reactivity patterns have sparked an immense interest in the chemistry of pyrrole derivatives. The general reactivity patterns of pyrrole are largely due to the compound being susceptible to electrophilic substitution. Figure 10 provides a general scheme for this concept.



**Figure 12.** Electrophilic substitution reactivity of pyrrole  
(*Modern Heterocyclic Chemistry*, **2011**, 269-375)

Electrophilic substitution predominantly occurs at the C2 ( $\alpha$ -position). Under the stipulation that an electron withdrawing group is present at the nitrogen or C2  $\alpha$ -position (**17a**) or when both positions are blocked, particular substituted pyrroles will also undergo reactions with electrophiles ( $E^+$ ) selectively at the C3 position (**17b**). As a result, electrophilic substitution can serve as a useful means for the elaboration of pyrrole derivatives. Attributable to the electron rich nature of most pyrroles, there is relatively low reactivity towards nucleophilic reagents. This reluctance to participate in nucleophilic substitution may be enhanced upon protonation or introduction of strong electron-withdrawing substituents. These reactions may occur with radical reagents or readily strong bases to produce pyrrolyl anion as shown in Scheme 1.



**Scheme 1.** Reactivity of pyrrole in nucleophilic substitution reactions (*Modern Heterocyclic Chemistry*, **2011**, 269-375)

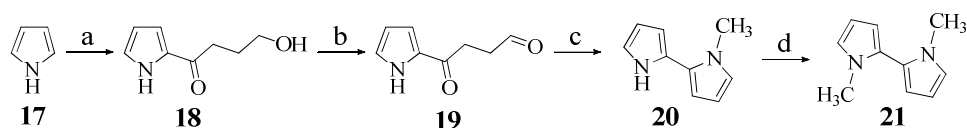
The reaction of pyrrole with strong bases allows the introduction of substituents at the nitrogen atom or respective carbon atoms. On the contrary, pyrroles with suitable N-blocking substituents are usually metallated at the C2 position which gives access to 2-substituted derivatives upon quenching with appropriate electrophiles. Restricted access to C2 can be accomplished by the regioselectivity of halogen-lithium exchange utilizing a bulky N-protecting

group with metallation of pyrroles at the C3 position. Incorporating these fundamental reactions in pyrrole ring synthesis offer practical tools in the preparation of a wide array pyrrole derivatives (Bergman and Janosik, 2011).

### 3.1 SYNTHESIS OF HALOGENATED DIMETHYL-2-2'-BIPYRROLES

Bipyrroles made their debut in *Chemical Abstracts* in 1914, with chemical synthesis beginning abruptly in the 1950s. Among the isomers of bipyrrole, the 2-2' bipyrrole motif is the most frequently reported in literature (Nikitin et al., 2007). The synthesis 2-2'-bipyrrole-based compounds, found in several marine organisms and other natural products reported in preceding studies, have become evident precursors for synthesis of the newly discovered halogenated dimethyl-2-2'-bipyrroles (Davis et al., 2001; Jolicoeur and Lubell, 2006; Pinkerton et al., 2007). Previous synthesis of 2-2'bipyrroles motifs have been achieved through several methods of synthetic reactions which includes, but are not limited to, metal-catalyzed coupling of iodopyrroles, Suzuki coupling, Paal-Knorr condensation, oxidative coupling, and reductive coupling reactions by use of Ulmann type reactions which are outlined and referenced by Fu and Gribble (Fu and Gribble, 2008b). Previous syntheses most widely use oxidative and reductive couplings to access the 2,2'-bipyrrole motif which have incurred undesirable side reactions. For example, oxidative coupling usually results in reactions of relatively low yields, and Ulmann type reactions usually require drastic conditions hindering direct access to sensitive substituents on the bipyrrole ring structure (Jiao et al., 2007). Because of these limitations, we adopted a method utilized by Fu and Gribble (2008b,2008c) to access our 2,2'-bipyrrole based compounds in a simple synthesis through exploration of the Paal-Knorr condensation reaction with slight

modification.

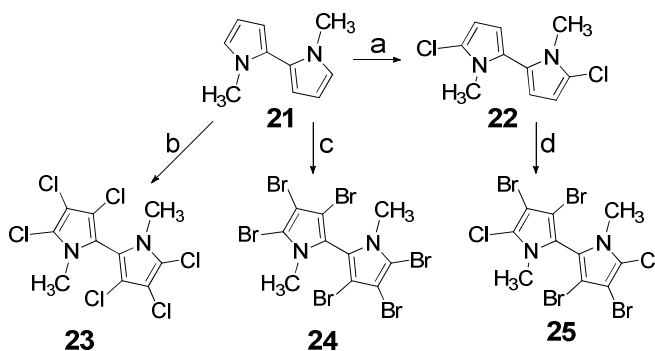


**Scheme 2** <sup>a</sup> Reagents and conditions: (a)  $\gamma$ -butyrolactone, toluene, Me-MgI, 55°C, 12-24 hr, 41% (two steps) (b) DCM, PCC, NaOAc, rt, 4 hr, 47% (c)  $\text{CH}_3\text{NH}_2\cdot\text{HCl}$ , toluene, MeOH, 60°C, 4 hr, 82 % (d) dry DMF, NaH, MeI, rt, 30 min, 95%

Pyrrole, **17**, was purchased and proved to be a relatively inexpensive starting material for exploration of our synthetic pursuits. Pyrrole was freshly distilled and subsequently reacted with methylmagnesium iodide and  $\gamma$ -butyrolactone in conditions first described by Nicolaou et al. (Nicolaou et al., 1985). The use of methylmagnesium iodide Grignard reagent as a base allows for the expedient deprotonation of pyrrole which subsequently reacts with  $\gamma$ -butyrolactone. As a result of being highly strained and unstable, the tetrahedral intermediate collapses and affords the pyrrole ketoalcohol, **18**, in decent yields (Scheme 2). Thin layer chromatography (TLC) analysis revealed that the crude product was relatively pure for next reaction and referencing provided accurate retention factor ( $R_f$ ) values. The primary pyrrole ketoalcohol (**18**) was subject to oxidation by pyridinium chlorochromate (PCC) in dichloromethane (DCM) to yield the desired pyrrole 1,4-diketoaldehyde, **19**, following a procedure described by Martin and Moody (1988).

In the interest of pursuing 2-2'-bipyrroles motifs, the Paal-Knorr condensation reaction proved successful in formulating the second pyrrole ring in 1-methyl-2-2'-bipyrrole, **20**. This reaction was discovered by Paal and Knorr more than a century ago and involves the reaction of primary amines with carbonyl compounds to give pyrroles (Li, 2004). In our reaction, 1,4-

diketoaldehyde, **19**, was reacted with methylamine hydrochloride which acts as a charged hydrogen ion intermediate in conditions well described by Fu and Gribble (2008b). The methylamine attacks the protonated carbonyl atom and generates 1-methyl-2,2'-bipyrrole, **20**. The methylation of **20** was proven to be successful in conditions described by Carmona et al. (1980). Sodium hydride (NaH) acts as a strong base to deprotonate the pyrrole. The electrophilic addition of iodomethane results in the formation of 1,1'-dimethyl-2,2'-bipyrrole, **21**, in 95% yield. This product was recovered as yellow oil and purified using silica gel column chromatography. <sup>1</sup>H- and <sup>13</sup>C-NMR spectroscopic analysis confirmed the structure of the 1,1'-dimethyl-2,2'-bipyrrole motif.



**Scheme 3** <sup>a</sup> Reagents and conditions: (a) dry THF, NCS, -78°C, 8 hr (b) dry THF, NCS, -78°C, 7 hr, (c) dry THF, NBS, -78°C, 7 hr, 93% (d) dry THF, NBS, -78°C, 7 hr

In order to pursue halogenated dimethyl-2,2'-bipyrroles series, we adopted a method employed by Gribble et al. (1999) with slight modifications. The dimethyl-2,2'-bipyrrole, **21**, was treated with excess N-Bromosuccinimide (NBS) or N-Chlorosuccinimide (NCS) to give the exhaustive chlorination and bromination of 1,1'-dimethyl-3,3',4,4',5,5'-hexachloro-2,2'-



bipyrrole, **23**, and, 1,1'-dimethyl-3,3',4,4',5,5'-hexabromo-2,2'-bipyrrole, **24**. The exhaustive bromination of **21** was successful and led to the desired product **24** in 93% yield. The mixed halogenated product, **25**, was generated in a reaction with equivalent amounts of NCS initially, followed by the subsequent reaction with excess NBS. Similar to the one pot reaction of Gribble et al. (1999) mixed halogenated product, the reactions from the exhaustive chlorination, **23**, and mixed halogenated product, **25**, led to a mixture of products as well. As noted by Gilow and Burton (1981), chlorination of some pyrroles with NCS in THF are not as selective as bromination due to increased molar amounts of NCS amplifies the occurrence of oxidation. Another explanation of the occurrence of mixed products maybe a result of a temperature and time issue. Regarding the NBS or NCS addition two methods have been employed. Method 1 involved cooling the reaction to -78°C and then adding the halogenation reagent. The reaction was allowed to proceed from -78°C to room temperature (rt) for 8 hours. Then, the work up of the reaction was completed upon reaching room temperature. Method 2 involved cooling the reaction to -78°C and then adding the halogenation reagent. The reaction was allowed to stir for 5 minutes and was immediately removed from the dry ice bath and stirred at room temperature for 7-8 hours. After several repeated experiments, Method 2 was selected. Structures and purity of the final halogenated compounds were confirmed through the use of NMR, high resonance mass spectrometry, and melting point analysis.

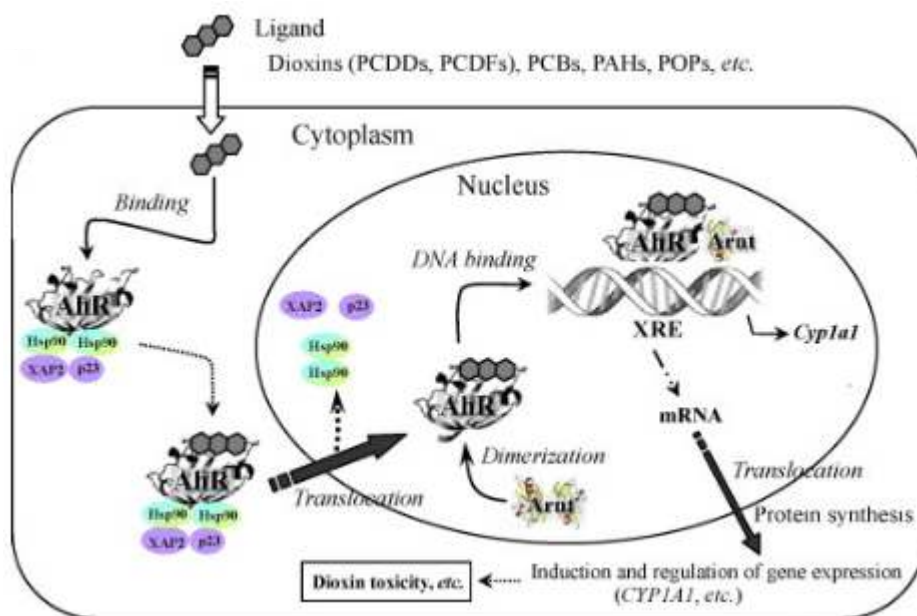
Successful synthetic pursuits of halogenated dimethyl-2,2'-bipyrroles led to the curiosity of these compounds having similar toxicity profiles as anthropogenic polychlorinated biphenyls (PCBs). The next chapter will discuss the significance of the biological activity of various halogenated pyrroles and their relationship with PCBs in a relevant marine fish species.

#### **IV. BIOLOGICAL ACTIVITY OF HALOGENATED DIMETHYL BIPYRROLES AND RELATED COMPOUNDS**

Data on the biological activity as well toxicological studies of HDBPs and related compounds are lacking. These types of studies are critical for the characterization of adverse effects these contaminants may pose to various biota. One study has evaluated the toxicological effects of these compounds on adult American kestrels (*Falco sparverius*) through HDBP-induced changes which includes reproductive and morphological endpoints (Tittlemier et al., 2003a). In the kestrel study, no significant dose-dependent effects were observed which suggests that HDBPs were not an acute threat to the reproductive and morphological development of the species tested. As stated in a previous chapter, responses to contaminant exposure is marked as a continuous series of biochemical and physiological changes in biota, and these changes may not be detected or reflected upon in nature through observation. One of the most collective examples of biochemical changes that are reflected upon exposure to common environmental contaminants is the induction of the metabolizing enzyme cytochrome P450 1A (CYP1A) gene.

The induction of CYP1A, a sub family of the monooxygenases, has served as a biomarker of exposure for environmental contaminants such TCDD, PCBs, PAHs, and other halogenated aromatic hydrocarbons (HAHs) in the last three decades. These classical environmental contaminants are aryl hydrocarbon receptor (AhR) ligands and model CYP1A inducers. These

chemicals bind effectively with the AhR and activate several genes, including CYP1A, and result in a series of conformational changes which the receptor binds to a particular DNA sequence increasing the production of the CYP1A enzyme.



**Figure 13.** Mechanistic model of the AhR signaling pathway. Adapted with permission from *Phytochemistry*, 2008, 69(18), 3117-3130

This AhR-mediated response can lead to acute and chronic toxicity from alteration of gene expression in susceptible cells (Denison and Nagy, 2003; Gonzalez and Fernandez-Salguero, 1998; Whyte and Jung, 2000). These biochemical changes can be used to monitor and draw conclusions about the toxicity from exposure to environmental contaminants. Efforts of the evaluation of biochemical changes of HDBPs and Q1, the mono-methylated bipyrrole derivative, have been exploited in two studies by Vetter et al. (2004) and Tittlemier et al (2003b). Although work with the biological activity of Q1 is still uncertain, evaluation of HDBPs congeners has revealed that three pure HDBP congeners (DBP-Br<sub>6</sub>, DBP-Br<sub>4</sub>Cl<sub>2</sub>, DBP-Br<sub>5</sub>Cl) and two mixtures

induce CYP1A in chicken embryo hepatocytes. What was interesting about the chicken hepatocyte study is that with all HDBP congeners and mixtures, an accumulation of porphyrin occurred at minimal levels of CYP1A activity. Accumulation of porphyrins leads to a condition called porphyria which disrupts the heme biosynthetic pathway. Many of the polyhalogenated aromatic compounds are potent porphyrinogenic chemicals and are inducers of the cytochrome P450 dependent monooxygenases (Safe and Hutzinger, 1984). Though the mechanism of porphyrin accumulation is relatively unclear, its accumulation may be directly or indirectly related to CYP1A. It was shown that CYP1A induction and/or AhR activation in porphyrin accumulation were mediated by HAHs that are planar, whereas the mechanism of porphyrin accumulation of nonplanar HAHs were not regulated by CYP1A induction (Lorenzen et al., 1997). In correlation with mammals and birds, HAHs that exhibit a planar moiety bind at higher affinities to AhR than their *non-ortho* substituted counterparts and *ortho* substituted are least effective at binding to AhR. Previous studies have shown that in the treatment of rats with *ortho* substituted or nonplanar PCBs are dominated by phenobarbitone type CYP2B induction which may also initiate toxicity through an oxidative stress mechanism and promote bilirubin degradation (De Matteis et al., 2002; Safe and Hutzinger, 1984).

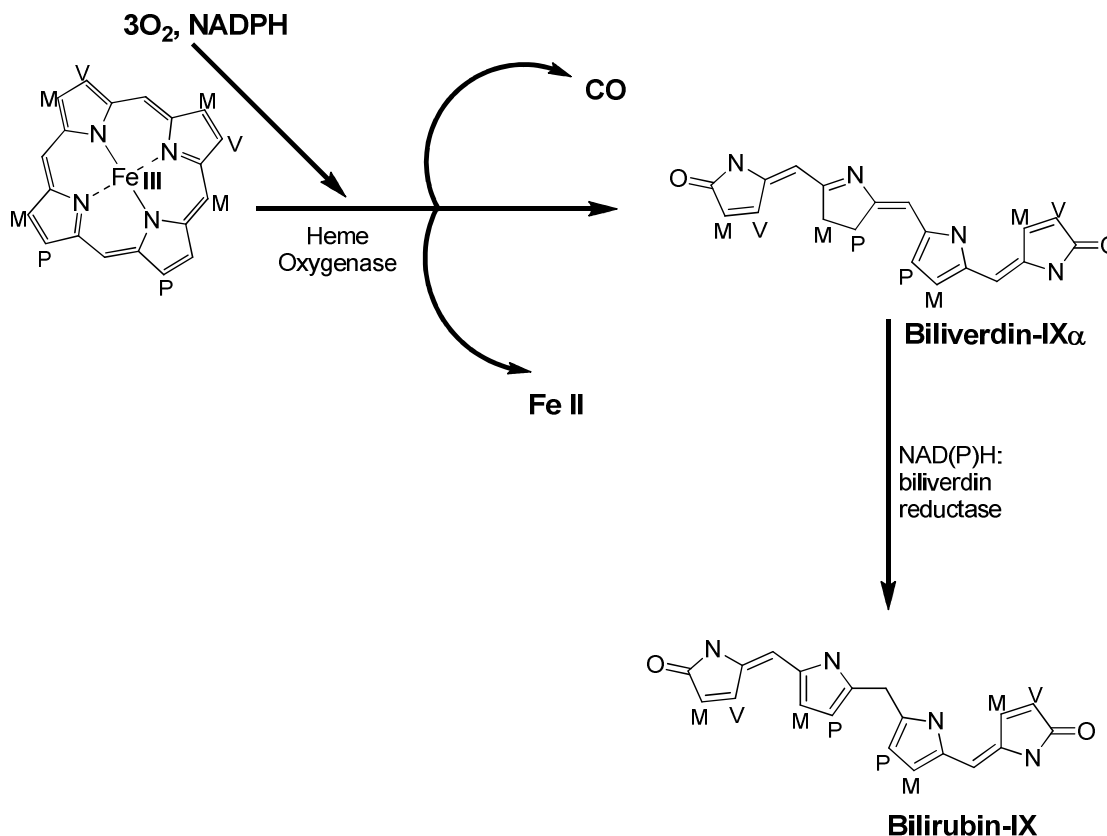
The work that explored CYP1A activity and porphyrin accumulation in chicken hepatocytes captivated a relation of HDBPs to anthropogenic halogenated aromatic hydrocarbons. However this study neither reflected the ability of HDBPs to induce CYP1A in a relevant wildlife species to adequately predict its toxicity in the environment nor did it sufficiently explain the accumulation of porphyrin. Based on these studies, we have focused our efforts on examining the effects of halogenated bipyrroles to inhibit heme oxygenase (HO), the

enzyme responsible for the catalytic oxidation of heme, which may account for the accumulation of porphyrins seen in the hepatocyte models. The key objectives of the research presented with in this thesis are to further characterize the biological activity of these compounds through use of heme oxygenase assays to explain porphyrin accumulation and examine the developmental toxicity of three HDBP's (DBP-Br<sub>6</sub>, DBP-Cl<sub>6</sub>, DBP-Br<sub>4</sub>Cl<sub>2</sub>) in a relevant marine species, *Fundulus heteroclitus* for the induction of the cytochrome P4501A (CYP1A) gene through activation of AhR signaling pathway.

#### 4.1 HALOGENATED DIMETHYL-2-2'-BIPYRROLES HEME OXYGENASE ACTIVITY ASSAY

Heme oxygenases catalyze the first and rate-limiting step in the oxidative breakdown of heme to form biliverdin-IX $\alpha$ , an open chain tetrapyrrole. HO activity mainly takes place in the liver or spleen, but its activity has also been detected in all organs and cell types. There are two active isoforms of HO in mammals, HO-1 and HO-2 (Kinobe et al., 2008). HO-1, fully characterized, is inducible by various stimuli and stressors being designated as heat shock protein-32 (HSP-32) (Takahashi et al., 2000). In this enzymatic reaction (shown in Figure 14), both HO enzymes (HO-1 and HO-2) oxidatively catalyze the conversion of heme to carbon monoxide (CO) and biliverdin-IX $\alpha$ . Biliverdin-IX $\alpha$  is subsequently converted to bilirubin-IX by an NAD(P)H-dependent reductase (Ryter et al., 2006). The products of HO activity, biliverdin and bilirubin, have strong antioxidant properties with a tremendous ability to scavenge free radicals and reduce oxidative stress. It is clear that HO/CO system plays a major role in cytoprotection and offers antioxidative benefits to organisms. CO and bilirubin are the major

players for cytoprotection and antioxidative benefits making HO an attractive therapeutic target for certain types of cancer and neurodegenerative diseases. The effect of HO activity is by and large to decrease the cellular concentration of toxicants and to increase the concentration of cell protectants (Kinobe et al., 2008).

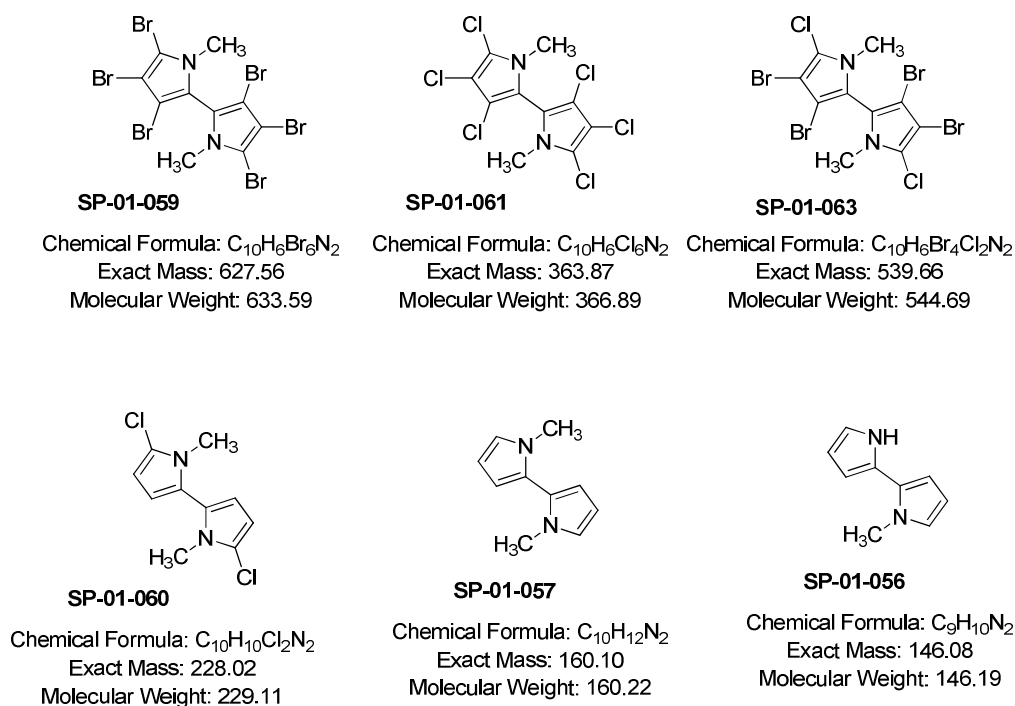


**Figure 14.** The enzymatic reaction of heme: Cleavage of heme, Fe III, releases coordinated ferrous iron and carbon monoxide (CO), to form the product biliverdin-IX $\alpha$  which is subsequently converted to bilirubin-IX (*Physiol. Rev.*, 2006, 86, 583-650)

The mechanisms and biologic effects involved in the HO/CO system are complex and beyond the scope of this discussion. In the previous study, the authors mentioned an accumulation of porphyrins in the chicken hepatocytes (Tittlemier et al., 2003b). The

accumulation of porphyrins seen in the chicken hepatocytes model sparked interest in the ability of HDBPs and its analogs to elicit HO inhibition. A decrease in HO activity may explain the accumulation of porphyrins observed by others because metalloporphyrins have been a major pharmacological tool in decreasing HO activity in studies referenced by Kinobe et al (Kinobe et al., 2008). To test this hypothesis we established collaboration with Professor David Stec at The University of Mississippi Medical Center, an expert in heme oxygenase, to test the HDBP analogs for HO inhibition.

#### 4.2 HEME OXYGENASE ACTIVITY ASSAY METHODS AND RESULTS

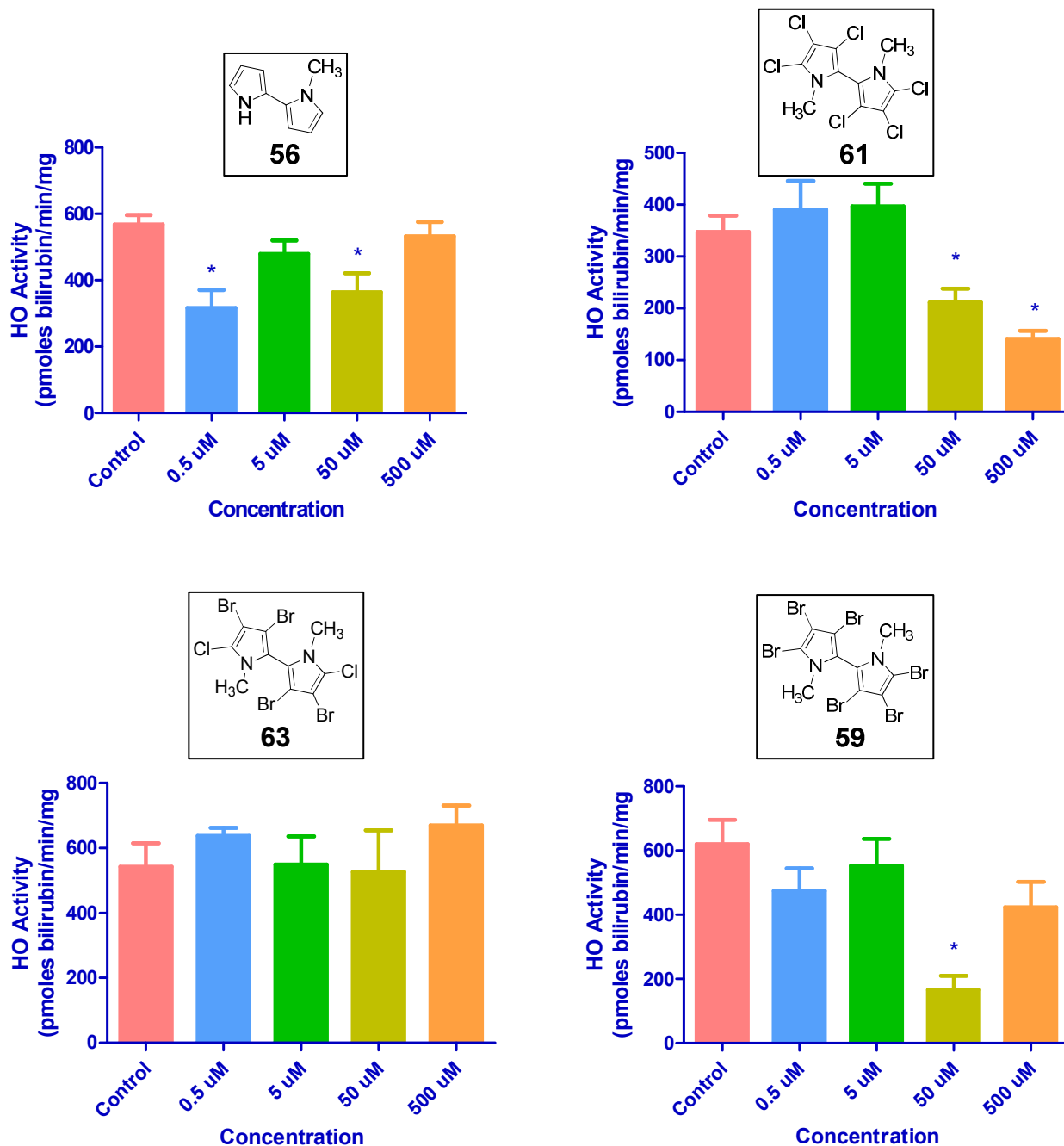


**Figure 15.** Halogenated dimethyl-2-2'-bipyrrroles (HDBPs) and synthetic intermediates submitted for testing for the inhibition of Heme Oxygenase-1

Halogenated dimethyl-2-2'-bipyrroles (HDBPs) and synthetic intermediates (Figure 15) were submitted to Professor David Stec for *in vitro* evaluation against heme oxygenase-1. Heme oxygenase assays were performed on liver lysates obtained from 16 week old male mice treated with the heme oxygenase-1 inducer, Cobalt protoporphyrin (CoPP, 50 mg/kg, i.p., Frontier Scientific, Logan, UT, USA) as previously described by Vera et al (2005; 2007). After 5 days mice were euthanized and liver collected and stored at -80°C until use. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the U.S. National Institutes of Health. Heme Oxygenase activity was determined by measuring the amount of formed bilirubin produced from hemin, expressed as picomoles of bilirubin formed per hour per milligram of microsomal protein. The amount of bilirubin formed was quantitated from the change in optical density ( $\Delta OD$ ) at 464-530 nm measured using an extinction coefficient of 40 mM/cm for bilirubin.

The results of HO activity in HDBPs are in Figure 16. After 1 h of treatment with the added substrate hemin (20  $\mu M$ ), significant inhibition of HO activity was found with HDBP congeners **59**, DBP-Br<sub>6</sub>, at 50  $\mu M$ , **61**, both at 50  $\mu M$  and 500  $\mu M$ , and the synthetic intermediate, **56**, at 0.5  $\mu M$  and 50  $\mu M$  concentrations. The mixed HDBP compound **63**, DBP-Br<sub>4</sub>Cl<sub>2</sub>, had no effect on HO activity. The results of this assay are not conclusive. During the course of this assay solubility issues were encountered; therefore, optimization of experiments is required. Subsequent data from this assay is still pending.

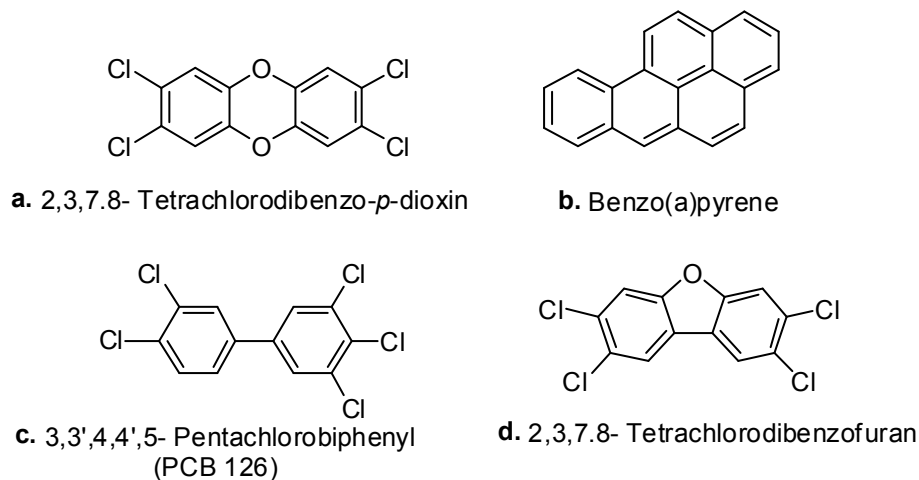




**Figure 16.** Inhibition of HO activity by HDBP congeners (**59**, **61**, **63**) and a synthetic intermediate (**56**); Cell lysates were treated with varying concentrations of compounds 30 min before exposure to hemin (20  $\mu$ M) and incubated for an additional 1h. HO activity was determined by measuring formed bilirubin expressed as picomoles of bilirubin formed per hour per milligram of microsomal protein; HO activity was analyzed by One Way Anova; n values ranged from 4-8. \*Significant difference from control ( $p < 0.05$ ).

#### HALOGENATED DIMETHYL-2,2'-BIPYRROLES ETHOXYRESORUFIN-*O*-DEETHYLASE (EROD) ACTIVITY ASSAY

Xenobiotic chemicals such as PCBs, polychlorinated dibenzo-*p*-dioxins, and dibenzofurans pose a global environmental health concern through various routes of exposure and the novel HDBPs are suspected to do the same. It is known that 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and other related chemicals some of which, shown in Figure 17, induce CYP1A through binding with the AhR signaling pathway to cause toxicity at significant exposure concentrations. Human health risks of exposures are more commonly estimated from exposures in animals (Silkworth et al., 2005). Receptor-mediated CYP1A induction by xenobiotic chemicals such as PAHs and other planar halogenated hydrocarbons are commonly established by ethoxyresorufin-*O*-deethylase (EROD) activity as an *in vivo* biomarker of exposure. EROD is a relatively sensitive indicator to exposure of contaminants in the uptake in fish and the induction of CYP1A has been particularly well established in these organisms. CYP1A induction has been closely related to detrimental effects such as apoptosis and developmental deformities such as cardiac edema, hemorrhage, anemia, and craniofacial malfunctions as seen in TCDD toxicity with zebra fish (Carney et al., 2004; Whyte et al., 2000). EROD activity is, at its best, a significant indicator of exposure to CYP1A inducers in organisms such as fish, but adverse effects in organisms at higher levels of organizations is questionable. Thus, using EROD activity as a biomarker of exposure in smaller organisms can prove useful in establishing the critical need for human health risk assessment studies.



**Figure 17.** Examples of AhR ligands

The most practical feature of monitoring CYP1A induction in the environment is the tendency of the enzyme to increase in concentration upon chemical exposure which leads to a typical dose-response curve. Examining the well characterized CYP1A induction models in fish, these enzymes are mainly concentrated in the liver but also can be detected in the kidney, gastrointestinal tract, gill tissue, and even in the urinary bladder (Varanasi, 1989). EROD activity in fish models provides a fingerprint of the presence of AhR-mediated compounds in a relatively simple assay. EROD activity describes the rate of CYP1A mediated deethylation of the substrate 7-ethoxyresorufin (7-ER) to form the product resorufin. Induction of CYP1A estimated from EROD activity does not designate that a toxic response will occur, but it does indicate the likelihood that AhR ligands will in fact induce biochemical changes (Varanasi, 1989; Whyte et al., 2000). The EROD activity assay was used to test three HDBP's, DBP-Br<sub>6</sub> (**59**), DBP-Cl<sub>6</sub> (**61**), and DBP-Br<sub>4</sub>Cl<sub>2</sub> (**63**) in the embryos of a marine relevant species, *Fundulus heteroclitus*. With HDBPs bioaccumulating in trophic marine environments in the Atlantic and Pacific Ocean, *Fundulus* will serve as the perfect model for the research at hand due the prevalence of the

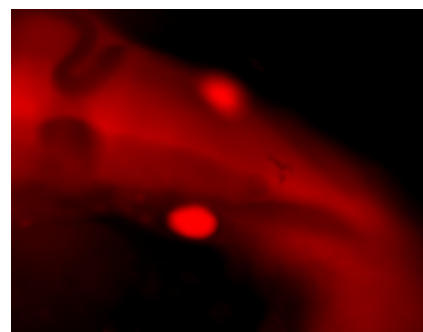
species in contaminated aquatic sites of the Atlantic Coast making them environmentally relevant for this current research. *Fundulus* are considered to be the “premier teleost model” in marine biology research, having several functionalized genes characterized specifically to this fish model capable of tolerating exposure to many environmental contaminants (Burnett et al., 2007). CYP450 enzymes are present, active, and inducible by halogenated hydrocarbons at low levels (Binder and Stegeman, 1984; Toomey et al., 2001). This fish model will help us investigate the developmental toxicity associated with exposure to relevant concentrations of HDBPs.

The advantages of using embryos in *in vivo* studies provide great benefit in investigating the toxicity of a contaminant. First spawning of adult fish can easily produce embryos in large numbers which are relatively inexpensive to maintain. Treated embryos can be maintained in small treatment volumes which reduce cost considerations when using expensive substance material. Developmental growth and specific organ toxicity can also be assessed visually and monitored daily due to the transparent nature of most fish embryos (Lillicrap, 2010). The rapid development and short life cycle of fish embryos allows a prompt evaluation of toxicity testing when using this model. *In vivo* studies can provide better insight on the prediction of the biological response in an intact organism and would give more feasible results when trying to foresee what actually takes place in the environment (Usenko et al., 2007).

#### 4.4 ETHOXYRESORUFIN-O DEETHYLASE (EROD) ACTIVITY ASSAY

##### METHODS AND RESULTS

Parental *Fundulus heteroclitus* (killfish or mummichog) were collected from an uncontaminated site near Beaufort, NC and were raised under the University Institutional Animal Care and Use Committee approved conditions and protocols. *In ovo* EROD investigations were conducted with slight modification of methods described by Nacci et al. (2005) and Wassenberg et al. (2002). *Fundulus heteroclitus* eggs were spawned from adult fish. *Fundulus* embryos were exposed to three HDBP congeners, PCB126 and a DMSO solvent control administered in a waterborne exposure. The embryos were exposed to the waterborne contaminants from 1 through 10 days postfertilization (dpf). Eggs were collected and fertilized by mixing with sperm and incubated for 20 minutes after fertilization. Embryos were then rinsed with artificial seawater (ASW). Fertilized eggs were allowed to develop for several hours and only embryos that appeared to have dividing cells were used for HDBP exposures. On the day of fertilization, 10 fertilized embryos were placed individually with 10 mL 22 ppt ASW with 3.6 µL of ethoxyresorufin per scintillation vial. Embryos were dosed with DMSO (solvent control), PCB126 (positive control) with a concentration of 500 ng/L, and three different HDBP congeners concentrations of 5, 50, 100 µg/L. Ten embryos were exposed to each of the HDBP congeners and PCB126 in a single scintillation vial for each treatment group and 3 replicate vials per treatment. Embryos were then allowed to develop in the vials and monitored daily for development and abnormalities.



**Figure 18.** *Fundulus* embryo exposed to PCB126 measured at 5 dpf

On 5 days post fertilization (dpf), embryos were analyzed for EROD activity by visualizing resorufin collected in the urinary bladder, as shown in Figure 18, using fluorescent microscopy. All images were captured using a Nikon DS-Qi1MC microscopy with epifluorescence and 10x magnification using a rhodamine red filter set. Fluorescence of the resorufin substrate accumulated in the bladder was quantified by ImageJ software (NIH, USA). Fluorescent density was measured by selecting a fixed pinhole area centered over one bladder lobe, normalized by subtraction of background fluorescence. *In ovo* EROD values are expressed as the correct total cell fluorescence (CTCF) which account for the mean fluorescence of the background deducted from the integrated density [CTCF= Integrated Density – (Area of selected cell X Mean fluorescence of background)] in a method adapted from Burgess et al (2010) and Gavet et al. (2010). Following *in ovo* EROD analysis, embryos were placed in clean ASW containing all HDBP or PCB treatments until 10 dpf. Experiments were performed in triplicates using different batches of embryos from the same parental stocks. Two embryos per vial (n=3) for each treatment was used for EROD analysis. Digital images of EROD assay at 5 dpf are shown in Appendix B.

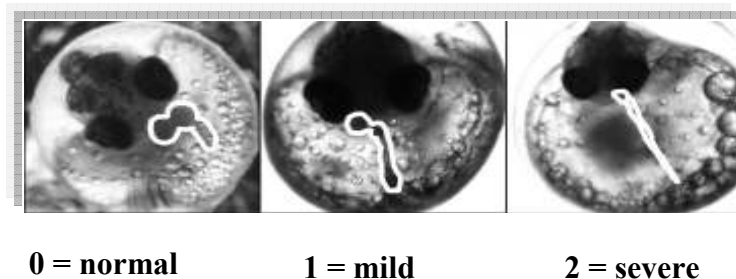
In this study, there was a significant difference in EROD induction between the PCB126 (500 ng/L) positive control, and the DMSO solvent control embryos ( $P < 0.0001$ ). There were no significant differences in EROD induction among the median (50  $\mu\text{g/L}$ ) and highest (100  $\mu\text{g/L}$ ) concentrations in the embryos exposed to HDBP congener **59** (DBP-Br<sub>6</sub>) and the control, but the lowest (5  $\mu\text{g/L}$ ) elicited EROD induction comparable to PCB126. HDBP congener **61** (DBP-Br<sub>4</sub>Cl<sub>3</sub>) elicited a significant difference in EROD activity from the solvent control only in 100  $\mu\text{g/L}$ , but the lowest and median concentrations of this congener were not statistically different

from the negative and positive controls. HDBP congener **63** (DBP-Br<sub>4</sub>Cl<sub>3</sub>) elicited a significant increase in EROD activity in all treatments. However, embryos exposed to 50 µg/L were significantly different from the HDBP **61** treatment groups. A two-way ANOVA was used to compare the corrected total bladder fluorescence among all three HDBP congeners. A Bonferroni post-tests were conducted to determine differences among concentrations between compounds (Figure 21). A significant interaction between HDBP congener dose responses indicates that the effect of treatment was different among HDBP congeners and concentrations ( $P < 0.05$ ). There were significant effects on EROD activity due to HDBP congeners ( $P = 0.004$ ) and concentration ( $P < 0.0001$ ). The Bonferroni post-tests revealed that HDBP congener **63** had significantly higher EROD activity compared to HDBP congener **61** at 5 and 100 µg/L and HDBP congener **59** at 100 µg/L.

#### 4.5 EMBRYO MORTALITY AND DEFORMITY ASSESSMENT

Embryos were allowed to develop in scintillation vials with respective treatments and monitored daily for development and abnormalities. Embryo mortality death was not statistically analyzed because few deaths occurred. Dead embryos were removed from a treatment group. Embryos were scored blind according to methods described by Meyer and Di Giulio (2002) with slight modifications. Embryos were scored for blood clot, edema, and tube heart on 10 dpf under magnification on a scale of 0-2 (normal, moderate, severe) for tube heart and a scale of 0-1 (present, not present) for blood clot and edema. After scoring each embryo, the number of embryos with the same deformity score was multiplied by the corresponding deformity score number. The numbers were added together and divided by the total number of embryos in each

vial to get mean deformity score. Experiments were performed in triplicates. Figure 19 demonstrates how the tube heart is scored.

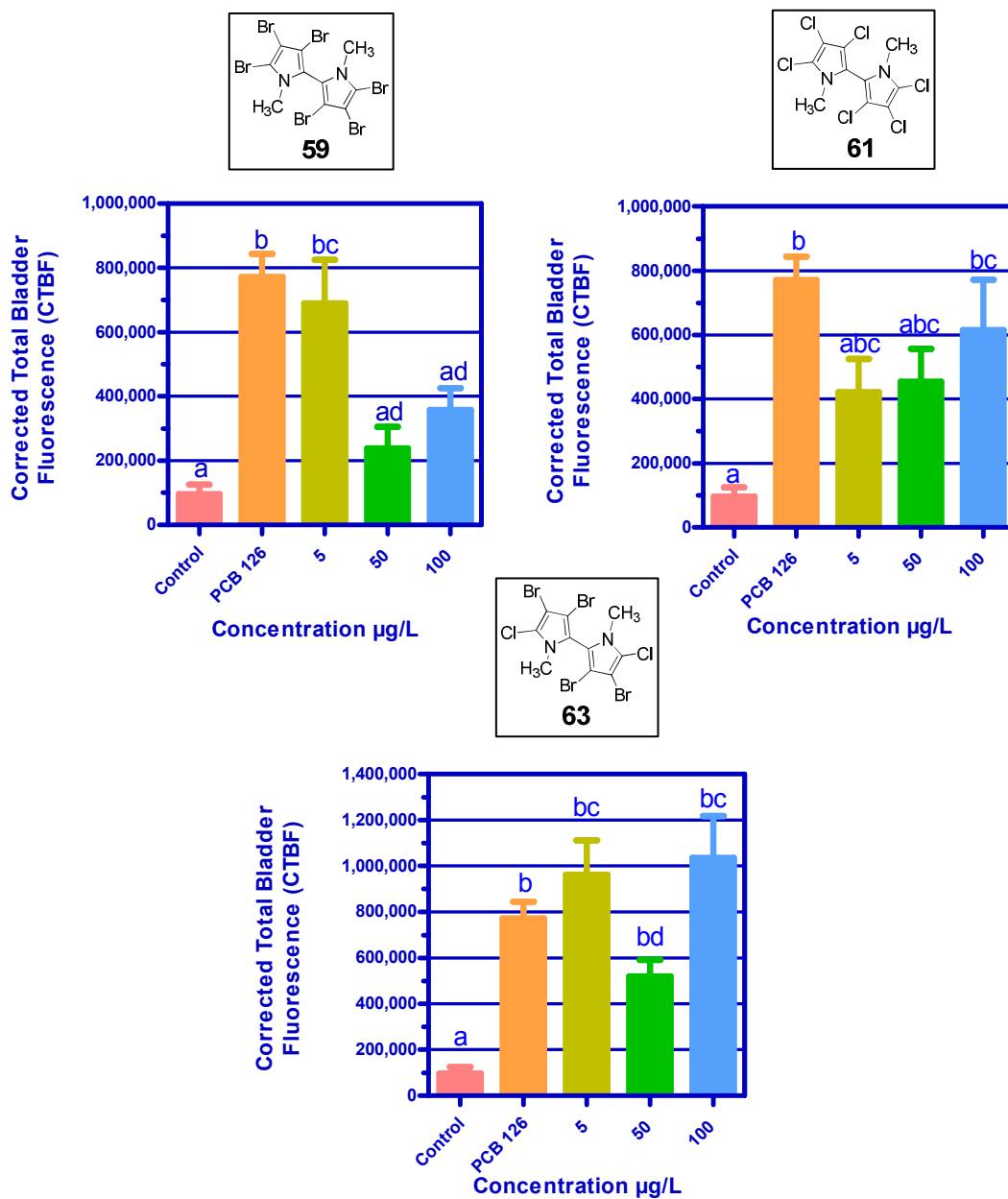


**Figure 19.** Morphological feature of embryos with a normal and tube heart; Adapted with permissions from *Aquat. Toxicol.*, **2008**, 87(4), 289-2295

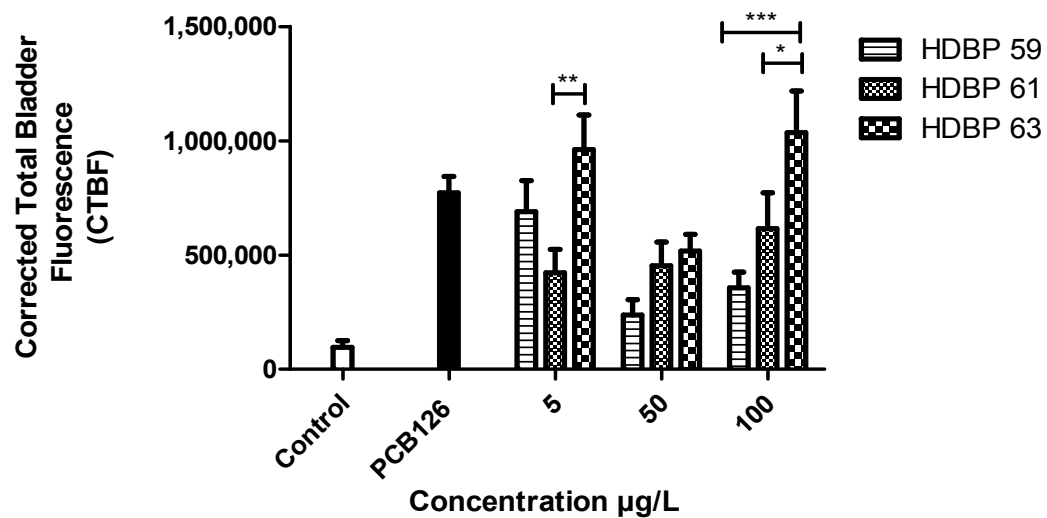
Results revealed that exposure to PCB126 and HDBP congeners elicited several types of deformities in

killifish embryos. The deformities observed occurred in heart as moderate to severe elongation noted as a tube heart with the presence of a blood clot. Severe deformities were observed in PCB126 (positive control) exposed embryos with a deformity index greater than 2. No deformities were observed in embryos exposed to HDBP congener **59**, DBP-Br<sub>6</sub>, at any concentration. Embryos exposed to HDBP congener **61**, DBP-Cl<sub>6</sub>, developed significant deformities only at 5 µg/L compared to control. Embryos exposed to HDBP congener **63**, DBP-Br<sub>4</sub>Cl<sub>2</sub>, developed significant deformities only at 50 µg/L.

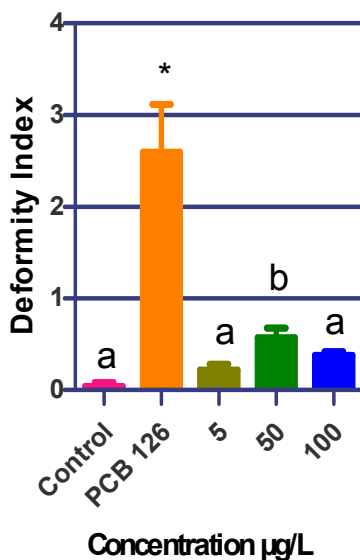
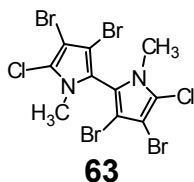
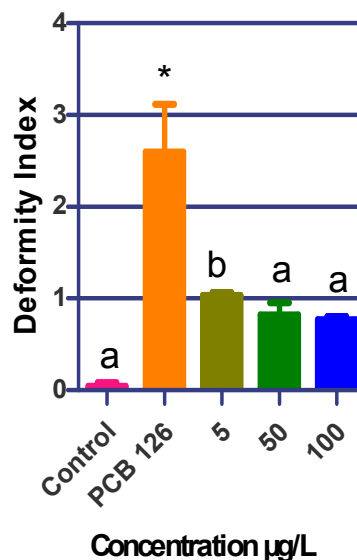
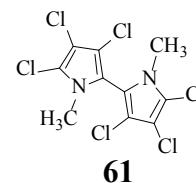
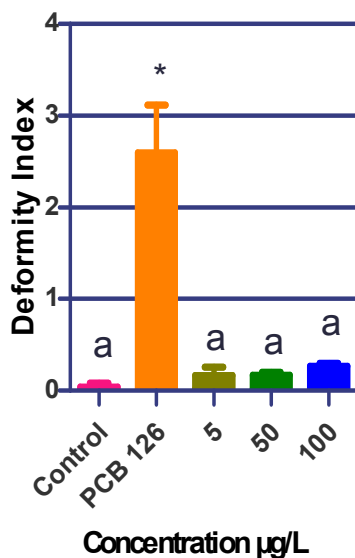
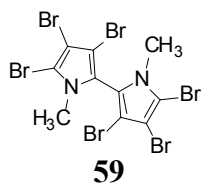




**Figure 20.** *In ovo* EROD activities in PCB126 (500 ng/L) and HDBP exposed embryos; EROD activity expressed as Corrected Total Bladder Fluorescence (CTBF) of embryo bladders; EROD induction was analyzed by One Way ANOVA; n=3; Histograms represent the mean CTBF  $\pm$  1SE. Note difference in scale on y axis.



**Figure 21.** EROD Activity expressed as Corrected Total Bladder Fluorescence of embryo bladders of PCB126 (500 ng/L) and HDBP exposed embryos at 5 dpf; EROD Activity was analyzed by two-way ANOVA followed by post hoc analysis; n = 3 vials per treatment; Error bars represent standard error of the mean; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Figure 22.** Deformity Index in PCB126 and HDBP exposed killfish embryos at 10 dpf; PCB126 embryos were exposed to 500 ng/L; Embryos were scored blind according to presence or extent of severity of blood clot, edema, and tube heart; Deformity Index were analyzed by nonparametric ANOVA;  $n = 3$  vials; 10 embryos/vial; Histograms represent the mean deformity index  $\pm$  1SE. \*Note PCB126 was excluded from the analysis as an outlier.

#### 4.6 DISCUSSION OF RESULTS

HO is an enzyme that degrades heme from aging red blood cells and gives rise to three products, CO, iron, and biliverdin. The HO-1 isoform of HO is inducible by various stimuli and stressors being designated as HSP-32 (Takahashi et al., 2000). In our study we discuss the ability of HDBPs and its analogs to elicit HO inhibition. Evaluation of whether inhibition of heme oxygenase is beneficial or detrimental for a particular organism is difficult to explain, but it has been noted that a reduced activity of heme oxygenase or the NAD(P)H-dependent reductase enzyme would result in a decreased heme degradation rate. Reflecting on the catalytic mechanism of HO, biliverdin is subsequently converted to bilirubin and a sustained inhibition of bilirubin formation may deprive the organism of the antioxidant activity endowed with bilirubin leading to oxidative damage and undesirable effects (Maines, 1988).

The heme oxygenase assays that were performed on liver lysates obtained from 16 week old male mice treated with the heme oxygenase-1 inducer, Cobalt protoporphyrin revealed significant inhibition of HO activity with HDBP congeners **59**, DBP-Br<sub>6</sub>, at 50 uM, **61**, both at 50 uM and 500 uM, and the synthetic intermediate, **56**, at 0.5 uM and 50 uM concentrations. It appears that only the HDBP congeners that are fully substituted with bromine or chlorine are inhibitors of HO, while the mixed halogenated compound, **63**, had no effect on HO activity. The lack of HO inhibition in HDBP **63** and at higher concentrations in other HDBP analogs may contribute to solubility issues.

It is well known that certain HAHs including PAHs and TCDD are extremely toxic to the early life stages of a variety of fish, avian, and mammalian species (Peterson et al., 1993). HAHs such as TCDD have been linked to embryotoxicity in several freshwater species (Toomey et al.,

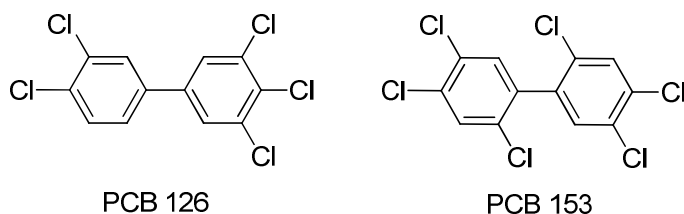
2001), but literature reports are scarce on the sensitivity of marine fish to HDBPs. Previously the ability of HDBPs to induce CYP1A was shown in chicken hepatocyte models. Here we show the ability of three HDBP congeners (DBP-Br<sub>6</sub>, DBP-Cl<sub>6</sub>, and DBP-Br<sub>4</sub>Cl<sub>2</sub>) to affect the development and CYP1A activity (measured as EROD induction) in the embryos of a marine relevant fish species *Fundulus heteroclitus*. *Fundulus* is a common estuarine fish that is widespread along the U.S Atlantic coast native to where HDBPs are being found (Toomey et al., 2001). This species serves as a valuable model for assessing developmental toxicology because the reproductive and development biology of this organism is well known and linked to other studies in its ability to adapt to changing environmental conditions (Armstrong and Child, 1965; Toomey et al., 2001).

Common environmental contaminants such as benzo(a)pyrene (BaP) and PCB126 are known to bind to AhR and initiate a downstream cascade of events that results in the upregulation of CYP1A activity which can be measured as EROD activity (Wassenberg et al., 2002). Upregulation of CYP1A can boost the metabolism of some compounds, such as BaP, to generate reactive intermediates to form DNA adducts, while other HAHs act as AhR agonists and cause oxidative stress (Alsharif et al., 1994). EROD activities have been used as a biomarker for exposure to contaminants in fish (Collier et al., 1995). Here we established that exposure to HDBP congener **61** dose-dependently statistically increased *in ovo* relative to control embryos. As expected, basal EROD activity was low in solvent control embryos not exposed to inducing chemicals. PCB126 exposed embryos reach maximal EROD activity at a lower concentration in comparison to HDBPs. Maximal induce EROD activities were not coherent with all HDBP congeners. HDBP congener **63** elicited significant increases in EROD activity at all

concentrations but does not show a typical dose response curve, while congener **61** only elicited a significant maximal EROD at the highest concentration of 100 µg/L compared to the DMSO control. The lowest and median concentrations of HDBP congener **61** show no significant differences between the DMSO control, PCB126 or among HDBP exposed embryos (5, 50, 100 µg/L). Maximal EROD induction at the low (5 µg/L) concentrations was observed for HDBP congeners **59** and **63**. The phenomenon of the lack of EROD induction at median and high concentrations of HDBP **59** unrelated to cytotoxicity because the deformity index of HDBP congener **59** is relatively low and comparable to the deformity index of the solvent control. As noted by Tittlemier et al. (2003b), some studies show that induced EROD activities reach a maximum and decrease at higher concentrations (Kennedy et al., 1995; Lorenzen et al., 1997). Reasons proposed for this may be due to the inducing compound causing inhibition of the deethylase reaction or mechanism based CYP1A inactivation. Whether this points towards EROD inhibition or CYP1A inactivation in our study as causes of decline of EROD activity would require immunoassay of the CYP1A protein. PCR analysis of the CYP1A gene expression in the embryos for this study has yet to be examined. Another explanation of the unusual significant EROD induction at lower concentrations in our study, maybe again due to solubility issues with certain HDBP congeners.

Possible mechanisms of action of HDBPs can be derived by comparing the toxic effects of the related isomers and congeners of the more toxic halogenated aromatics such as TCDDs and PCBs. The responses to AhR ligands can vary with dose and time of exposure, but the toxicity to individual HAHs can also be structure dependent as with the congeners of PCBs (Bemis et al., 2005; Sanchez-Alonso et al., 2003). PCBs with one or no chlorine substitution in

the *ortho* position (PCB126 and PCB77) can assume the planarity and go on to elicit TCDD like toxicity by binding to the Ah receptor. PCBs with substantial chlorination in the *ortho* position are considered nonplanar PCBs (PCB95 and



**Figure 23.** Coplanar PCB126 and nonplanar PCB153

PCB153). These compounds are sterically hindered and TCDD like properties disappear. Sanchez-Alonso et al. demonstrated that both a nonplanar PCB153 congener and a coplanar PCB77 congener can both induce apoptosis correlated with the loss of cell growth in neuronal cell cultures, but the extent of apoptosis was greater for the coplanar PCB congener because the compound was more cytotoxic. The neuronal cell apoptosis study concluded that PCB induce apoptosis due to a difference in molecular mechanisms that are involved in the induction of apoptosis by the degree of planarity by individual PCB congeners (Sanchez-Alonso et al., 2003).

Anthropogenic planar HAHs and coplanar PCBs are potentially harmful to aquatic organisms and accumulate in fish tissues and are maternally transferred to offspring. Planar HAHs interact additively to cause developmental abnormalities in fish embryos such as blue sac disease, edema, hemorrhages, craniofacial, malformations, and impaired growth and survival in conjunction with induction of CYP1A enzymes. Coplanar PCBs cause uncoupling of CYP1A and go on to cause generation of reactive oxygenated species and oxidative stress in fish which eventually leads to cell death (Couillard et al., 2011; Schlezinger et al., 2006).

The question to whether involvement of CYP1A induction acts as a contributing factor to embryo mortality and elicitation of deformities in HDBPs is still unclear. The concentrations of

HDBPs examined here did not cause any embryo mortality. Given how embryos were scored for deformities, the highest score for an embryo with extreme deformities would be given a deformity index of 4. A slight yet significant increase occurred with the deformity index of HDBP congener **61** at 5, 10, 100 µg/L and **63** at 50 µg/L with the observed effects of moderate tube heart, blood clots, and few incidences of edema in some embryos which more severe deformities occurred at a low concentration (500 ng/L) occurred with PCB126 exposed embryos. Perhaps repetition of the assay on embryos at more developmental stages would clarify if CYP1A induction is directly related to mortality or incidences of abnormality through differential stages of development as mentioned by Toomey et al. (2001) in comparison of the effect of CYP1A induction on different developmental stages and apoptosis of *Fundulus* embryos upon exposure to TCDD.

As mentioned previously that some HAHs and PCBs are planar and coplanar in the way they effect CYP1A activity, HDBPs are predicted to have a nonplanar configuration being fully substituted with bromines or chlorines stated by Tittlemier et al. (2003b). If HDBPs indeed adopt a nonplanar configuration this may explain their effect on EROD activity and their mechanism of action. These compounds may bind weakly to AhR to induce CYP1A activity with little or no toxicity and may involve different molecular mechanisms similar to some nonplanar HAHs.



## **V. CONCLUSTIONS AND FUTURE DIRECTIONS**

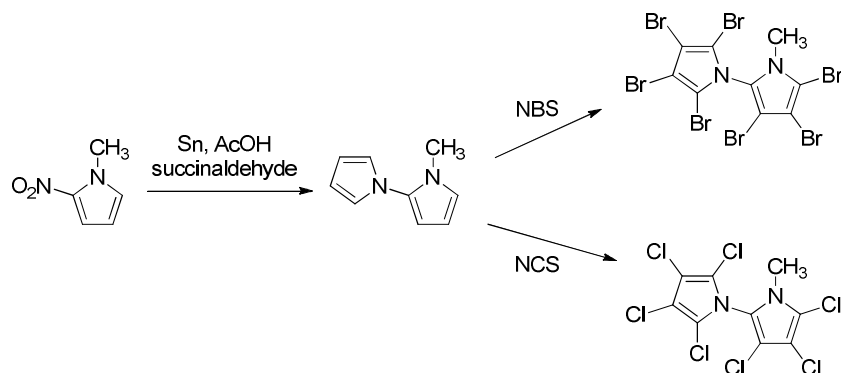
Whether HDBPs are of natural or anthropogenic origin has long been a debate among researchers since they have were first detected in marine samples in the Pacific and Atlantic Ocean. Although evidence points towards more of a natural source, the natural producers of these compounds have not been identified. The debate of what source these compounds may have originated has lessened over recent years and focus has shifted towards toxicological investigations. It is clear that these compounds are prevalent among marine samples, bioaccumulating and causing contamination among birds, cetaceans, and fish. Even with the extent of contamination of HDBPs and similar compounds being far-reaching in places such as the Antarctic, no toxic effects could be determined in environmental samples with the highest concentrations (Vetter, 2006).

CYP1A activity (EROD) is an excellent biomarker of exposure for contamination among fish (Collier et al., 1995). Studies have suggested that generation of reactive oxygenated species and oxidative stress in fish are associated with induced CYP1A which have occurred in coplanar PCBs (Couillard et al., 2011; Schlezinger et al., 2006). The fact that HDBPs may exhibit a nonplanar configuration is striking and may explain the way in which these compounds respond to AhR because PCBs and HAHs known to induce CYP1A are either coplanar or planar. In our study, three HDBP congeners (DBP-Br<sub>6</sub>, DBP-Cl<sub>6</sub>, DBP-Br<sub>4</sub>Cl<sub>2</sub>) were effective at inducing

EROD activity at  $\mu\text{g/L}$  concentrations comparable to PCB126, but unlike PCB126 only slightly elicited deformities with no mortalities. The significance of CYP1A, measured as EROD, suggests that HDBPs may indeed exhibit AhR-dependent toxicity. HDBP congener **61** appears to incorporate the typical dose response curve and HDBP congener **63** elicited significant EROD induction at all concentrations. Though the assessment of deformities in this study was not comparable to dioxin like effects, the association of CYP1A induction in relation to specific toxic effects is vague. Repetition of this assay may clear this up, but examining HDBPs at higher concentrations maybe problematic because the solubility of these compounds is very limited. Future needs of this study should consider immunoassay of the CYP1A protein or PCR analysis to adequately assess CYP1A's role in examining the biological activity in *Fundulus*.

Though disruption of the AhR signaling pathway seemed to explain the CYP1A induction seen in chicken hepatocyte models described by Tittlemier et al. (2003b), the authors were unable to explain the porphyrin accumulation. Within the current study we have shown halogenated bipyrroles to elicit HO inhibition, which likely accounts for the porphyrin accumulation observed by others. To our knowledge this is the first report of HDBPs as HO-1 inhibitors. It appears that only the HDBP congeners that are fully substituted with just bromines or chlorines are inhibitors of HO, while the mixed halogenated compound, DBP-Br<sub>4</sub>Cl<sub>2</sub>, had no effect on HO activity. Further in-depth studies are needed to fully elucidate the mechanism of HDBPs and its analogs on the attenuation on HO-1 activity.

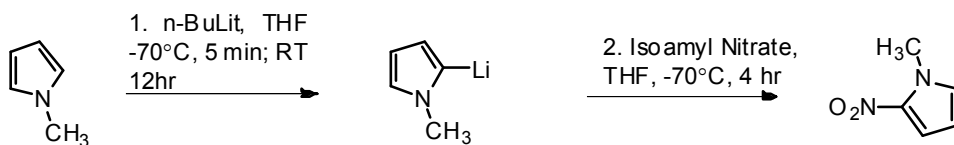
## 5.1 SYNTHETIC CONSIDERATIONS



**Figure 24.** Published route of 1,2'-bipyrrroles by Fu and Gribble, *Organ. Prep. & Proc. Int.*, **2008**, 40(6), 561-566

Given that the HDBPs used in this study were halogenated dimethyl-2-2'-bipyrrroles, it is a matter of curiosity of whether 1,2'-bipyrrroles would also inhibit heme oxygenase or induce CYP1A because these compounds are also among marine environmental samples ranging from 3-14,000 ng/g lipids (Vetter, 2006). A published route to 1,2'-bipyrrroles were previously synthesized by Fu and Gribble by use of a nitropyrrole and succinaldehyde (Fu and Gribble, 2008a). We wanted to pursue making these 1,2'-bipyrrroles. The nitropyrrole from the published route in Figure 24 proved to be quite expensive, so efforts turned to finding alternate routes to synthesize the 1-methyl-2-nitropyrrole.

An alternate route to 1-methyl-2-nitropyrrole was adapted from Brittain et al. (1982) by C-metallation of 1-methylpyrrole using n-butyl lithium to yield 2-lithiopyrrole which was subsequently reacted with a suitable electrophile. After several attempts, this reaction yielded undesired side reactions; therefore, efforts towards this route were discontinued.



**Scheme 4** Attempt at 1-methyl-2-nitropyrrole

Nitrations with pyrroles have proved to be dangerous especially with the use of fuming nitric acid and have yielded complex mixtures with a substantial amount of decomposed materials in other nitrating systems. Another attempt towards 1-methyl-2-nitropyrrole was made through the mononitration of pyrrole to yield 2-nitropyrrole in hopes of methylating the pyrrole ring. A method adapted from Tanemura et al. (2003) made use of an aromatic nitration with cerium (III) ammonium nitrate with acetic anhydride ( $\text{Ac}_2\text{O}$ ). Using this system as nitrating reagent will yield aromatic nitrations in relatively mild conditions compared to the frequently used  $\text{HNO}_3$ - $\text{Ac}_2\text{O}$  nitrating system for acid sensitive or active substrates which require low cooling temperatures due to intense exothermic reactions. Pyrrole is a very active compound and requires upmost attention for overheating when trying to pursue nitrations. Taking a look back at efforts using this mild reagent system, this reaction may require a reaction time of 24+ hrs in order to get this nitration to occur on the pyrrole ring.

## 5.2 CLOSING THOUGHTS

In this study we wanted to encompass the synthesis and biological evaluation of HDBPs, a class of naturally occurring metabolites that closely resemble anthropogenic pollutants like PCBs, PBBs, and PBDEs. There is no record of the industrial synthesis or manufacture of these compounds though they do exhibit structural similarity to the marine natural bacterial

hexabromo-2,2'-bipyrrole and may point towards a biogenic source (Andersen et al., 1974). It is important to note that HDBP congeners studied here and the other 1,2'-bipyrroles we attempted to pursue are prevalent and circulating in the marine environment. Many halogenated marine products are thought to play a role as chemical defense agents against natural predators and this may suggest ways HDBPs bind to AhR (Paul et al., 1993).

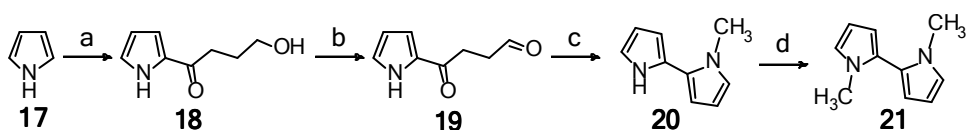
We were successful at synthesizing and structurally characterizing three target HDBP congeners, DBP-Br<sub>6</sub>, DBP-Cl<sub>6</sub>, DBP-Br<sub>4</sub>Cl<sub>2</sub>. Design and execution of an exposure analysis using the classical *Fundulus* embryo model using these three HDBPs revealed that these compounds were effective at inducing EROD, indicative of CYP1A induction. To our knowledge this is the first report of HDBPs used in an exposure analysis with an environmentally relevant marine fish species. If these compounds are indeed of biogenic origin then they will represent a class of naturally occurring AhR ligands as suggested previously (Tittlemier et al., 2003b). Our findings stress the importance of further characterizing the biological evaluation of HDBPs in an environmentally relevant species to investigate the risk of these compounds may pose to wildlife and human health.

In continuing our biological evaluation of HDBPs to explain porphyrin accumulation seen by others, we established HDBPs as HO inhibitors. HO is a class of enzymes that provides antioxidative benefit to organisms by catalyzing the oxidative breakdown of heme into biliverdin, CO, and ferrous iron. Bilirubin and CO are both powerful cytoprotectants against oxidative stress, making HO an attractive therapeutic target for certain types of cancer and neurodegenerative diseases. To our knowledge, this is the first report of HDBPs inhibiting HO. If we can further establish more halogenated bipyrrole compounds in HO inhibition and the

mechanisms involved, then these compounds will represent a new class of HO inhibitors. In conclusion, HDBP congeners **61** and **63** appear to be the leading compounds in EROD induction and HDBP congener **61** in HO activity in continuing future toxicological investigations regarding of the biological activity of HDBPs.

## VI. EXPERIMENTAL

### Preparation of 21 (Scheme 2):



**Scheme 2** <sup>a</sup> Reagents and conditions: (a)  $\gamma$ -butyrolactone, toluene, Me-MgI, 55°C, 12-24 hr, 41% (two steps) (b) DCM, PCC, NaOAc, rt, 4 hr, 47% (c) CH<sub>3</sub>NH<sub>2</sub>•HCl, toluene, MeOH, 60°C, 4 hr, 82 % (d) dry DMF, NaH, MeI, rt, 30 min, 95%

### Pyrrole ketoalcohol (18):

Using an adapted method from Nicolaou et al. (1985), Purchased pyrrole, **17** (28 mL, 404 mmol), was freshly distilled and slowly added to 1.87M H<sub>3</sub>CMgI in Et<sub>2</sub>O (174 mL, 325 mmol) in anhydrous toluene (240 mL). Reaction stirred for 30 min at 55°C before  $\gamma$ -butyrolactone (27 mL, 354 mmol) was added dropwise to the reaction mixture. The reaction mixture continued to stir at 55°C for 12-24 hrs before being cooled to RT and diluted with saturated aqueous NH<sub>4</sub>Cl and DCM. The pH was adjusted to 6 using HCl and worked up in DCM/H<sub>2</sub>O. Organic extracts were separated twice using column chromatography in 100% Et<sub>2</sub>O to give crude **18**. Crude has a residual starting material and was subjected to the next step. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  7.20 (s, 2H), 6.17-6.09 (m, 2H), 3.57 (t,  $J$  = 6.1 Hz, 2H), 3.49 (s, 1H), 2.82 (t,  $J$  = 7.2 Hz, 2H), 1.91-1.80 (m, 2H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  178.42, 170.899, 118.94, 112.96, 68.71, 61.02,

30.82, 27.66, 27.09, 21.96; DEPT-135 (100 MHz, CDCl<sub>3</sub>)  $\delta$  118.94, 112.96, 68.71, 61.027, 30.820, 27.66, 27.096, 21.96.

#### **1,4-diketoaldehyde (19):**

Using an adapted method from Martin and Moody (Martin and Moody, 1988), **18** (2.42 g, 15.8 mmol, 1 eq) in dry DCM (100 mL) was added to a mixture of PCC (5.18 g, 24 mmol, 1.5 eq) with NaOAc (480 mg, 5.8 mmol) in DCM (250 mL) and stirred at RT for 4 hrs under argon before being filtered with through celite and washed with DCM. The filtrate was washed with H<sub>2</sub>O and combined extracts were dried Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated *in vacuo*. Crude material was purified on silica (100% Et<sub>2</sub>O) to give **19** as an orange solid. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  9.68 (s, 1H), 7.22 (s, 2H), 6.23-6.13 (m, 2H), 3.01 (t, *J* = 6.3 Hz, 2H), 2.78 (t, *J* = 6.3 Hz, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  200.15, 169.21, 118.969, 113.182, 37.49, 26.83; DEPT-135 (100 MHz, CDCl<sub>3</sub>)  $\delta$  200.15, 118.96, 113.18, 37.49, 26.83.

#### **1-methyl-2-2'-bipyrrole (20):**

Using an adapted method from Fu and Gribble (2008b), toluene (400 mL) and MeOH (100 mL) was added to a mixture of **19** (62 mg), CH<sub>3</sub>NH<sub>2</sub>•HCl (15.57 g), NaOAc (19.32 g) and stirred under air at 60°C for 4 hrs. Reaction mixture was concentrated *in vacuo* and washed with 1 N aq HCl/DCM. Organic extracts were retained and purified using column chromatography (100% Et<sub>2</sub>O) to give **20**. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.23 (s, 1H), 6.98 (d, *J* = 1.5 Hz, 2H), 6.67 (d, *J* = 3.0 Hz, 1H), 6.53 (dd, *J* = 3.4, 1.6 Hz, 1H), 3.89 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  127.56, 124.70, 123.51, 118.34, 109.55, 108.00, 107.15, 107.11, 35.28. DEPT-135 (100 MHz,

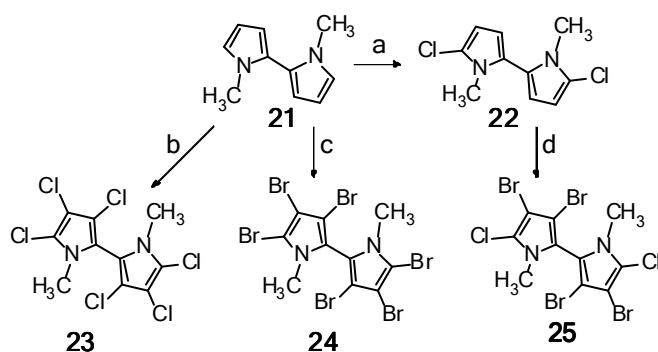


$\text{CDCl}_3$ )  $\delta$  123.51, 118.34, 109.55, 108.00, 107.15, 107.11, 35.28.

### Dimethyl-2,2'-bipyrrole (**21**):

Using a method from Carmona et al., (1980) a solution of **20** (1.06 g) and freshly distilled DMF (28 mL) was added dropwise into a solution of 60% NaH on mineral oil (1.03 g) and DMF (12 mL). The reaction mixture was stirred at RT under argon for 45 min before addition of  $\text{CH}_3\text{I}$ . The reaction mixture continued to stir for an additional 30 min before being quenched with water. The crude mixture was extracted with EtOAc. Organic extracts were rinsed with water and concentrated *in vacuo*. Purification on silica (10% EtOAc/Hexane) gave **21** as a yellow oil.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.00 (s, 2H), 6.51 (dt,  $J = 3.4, 2.6$  Hz, 4H), 3.78 (s, 6H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  125.39, 122.92, 110.78, 107.77, 34.58; DEPT-135 (100 MHz,  $\text{CDCl}_3$ )  $\delta$  122.90, 110.77, 107.76, 34.58.

### Preparation of 23-25 (Scheme 3):



**Scheme 3** <sup>a</sup> Reagents and conditions: (a) dry THF, NCS,  $-78^\circ\text{C}$ , 8 hr (b) dry THF, NCS,  $-78^\circ\text{C}$ , 7 hr, (c) dry THF, NBS,  $-78^\circ\text{C}$ , 7 hr, 93% (d) dry THF, NBS,  $-78^\circ\text{C}$ , 7 hr

**DBP-Br<sub>6</sub> (24):**

A method employed by Gribble et al. (1999) was adopted with slight modifications in order to pursue halogenated dimethyl-2-2'-bipyrroles series. **21** (200.8 mg) in dry THF (16 mL) was stirred under argon from RT to -78°C before addition of NBS (1.60 g). Ice bath was removed after 5 min and reaction mixture was stirred at RT for 7 hr. Reaction mixture was rinsed with DCM/H<sub>2</sub>O. Organic extracts were collected and washed with water and concentrated *in vacuo*. Crude was presented as orange solid and purified on silica (5%EtOAC/Hexane). Product was presented as white solid and recrystallized from hot DCM and slow evaporation of solvent. mp 242°C (decomp.) <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 3.49 (s, 6H); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 122.22, 106.79, 103.35, 101.25, 35.38; DEPT-135 (126 MHz, CDCl<sub>3</sub>) δ 35.38. Elemental Analysis Calcd for C<sub>10</sub>H<sub>6</sub>Br<sub>6</sub>N<sub>2</sub>: C, 19.13; H, 0.71; N, 4.37.

**3,5'-dichloro-1'-methyl-1,2'-bipyrrole (22):**

The same procedure employed in the synthesis of **24** was followed using **21** (177 mg) and dry THF (11 mL). Reaction mixture was stirred from RT to -78°C before addition of NCS (296 mg). Reaction mixture stirred at -78°C for 5 min before removal of ice bath. Reaction mixture was stirred at RT for 8 hr. Crude product was purified on column (100% Hexane – 50% EtOAC/Hexane). Product was presented as yellow oil and subjected to the next step.

**DBP-Cl<sub>6</sub> (23):**

The same procedure employed in the synthesis of **24** was followed using **21** (139.3 mg) and dry THF (11 mL). Reaction mixture was stirred from RT to -78°C before addition of NCS (296 mg).

Reaction mixture stirred at  $-78^{\circ}\text{C}$  for 5 min under argon before removal of ice bath. Reaction was then stirred at RT for 7 hr. Crude product was purified on column (100% Pet. Ether) and recrystallized from evaporating DCM. Pure product was presented as colorless crystals. mp  $242^{\circ}\text{C}$  (decomp.). Elemental Analysis Calcd for  $\text{C}_{10}\text{H}_6\text{Cl}_6\text{N}_2$ : C, 32.74; H, 1.35; N, 7.58.

**DBP- $\text{Br}_4\text{Cl}_2$  (25):**

The same procedure employed in the synthesis of **24** was followed using **21** (85 mg) and dry THF (12 mL). Reaction mixture stirred at  $-78^{\circ}\text{C}$  for 5 min under argon before removal of ice bath. Reaction was then stirred at RT for 7 hr. Reaction mixture was quenched with water and diluted with copious amounts of DCM. Organic layer was washed with water and concentrated *in vacuo*. Crude was purified on silica (10% EtOAc/Hexane) and recrystallized from DCM. Pure product was presented as white crystals. mp  $226^{\circ}\text{C}$  (decomp).



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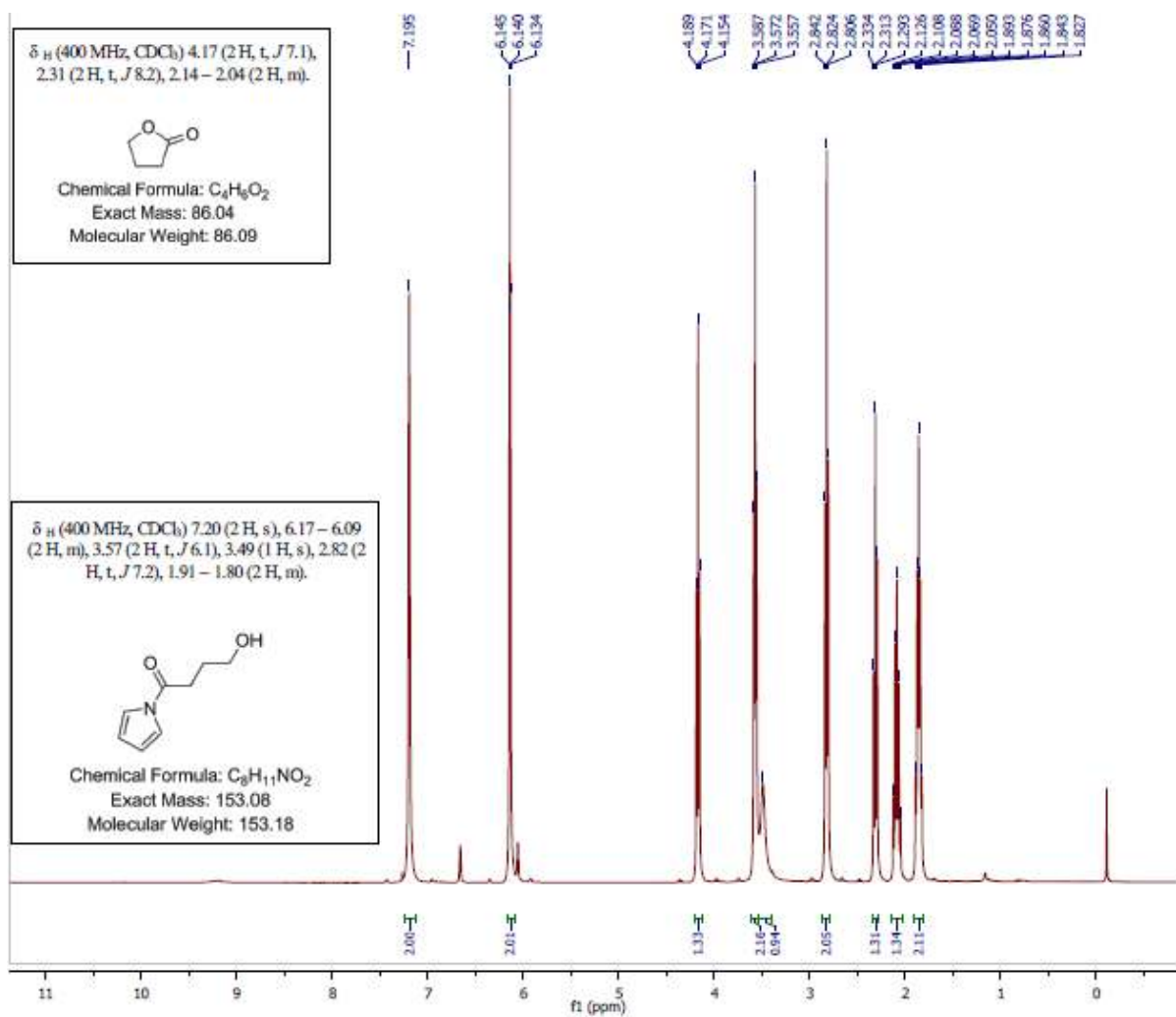
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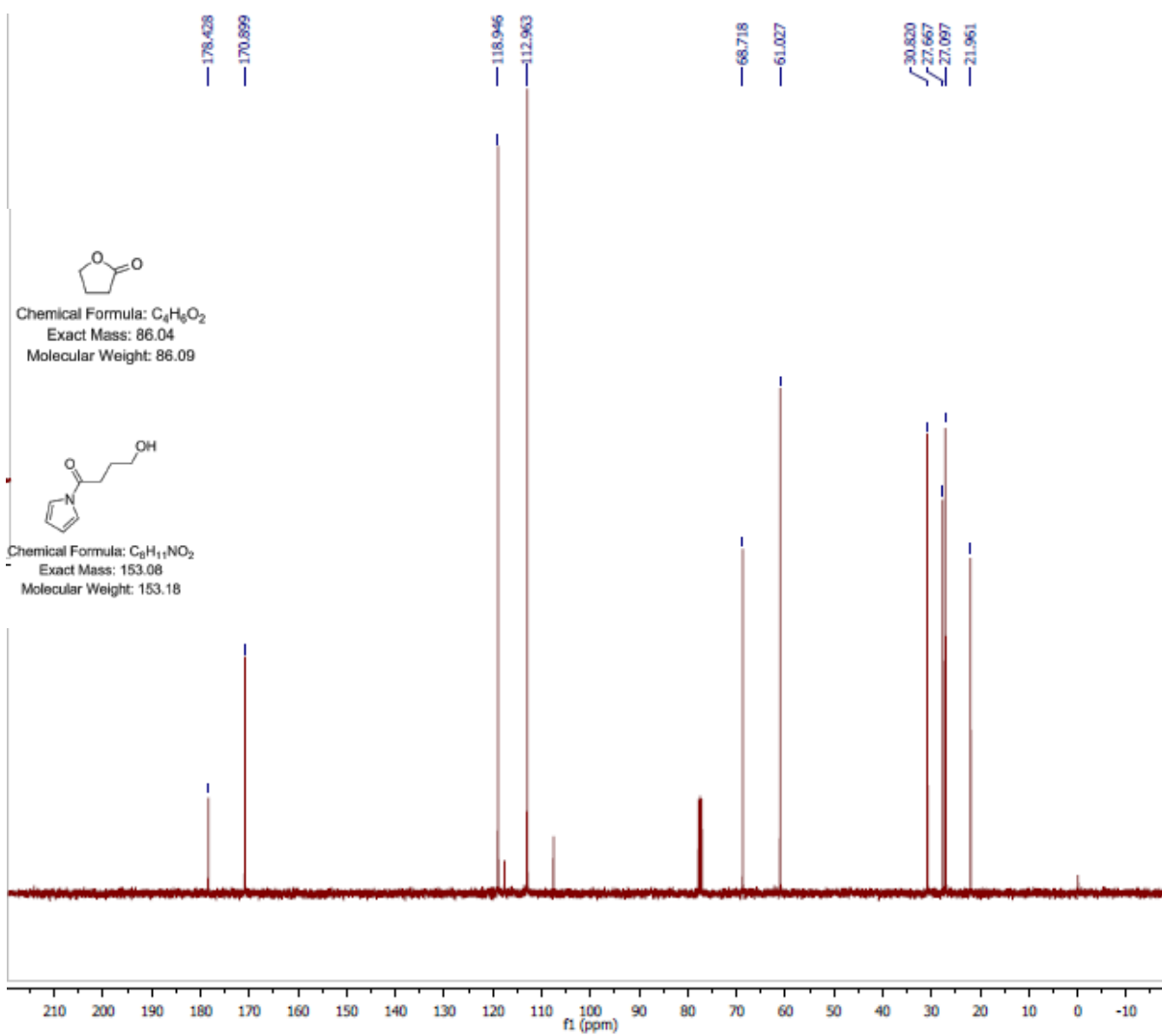
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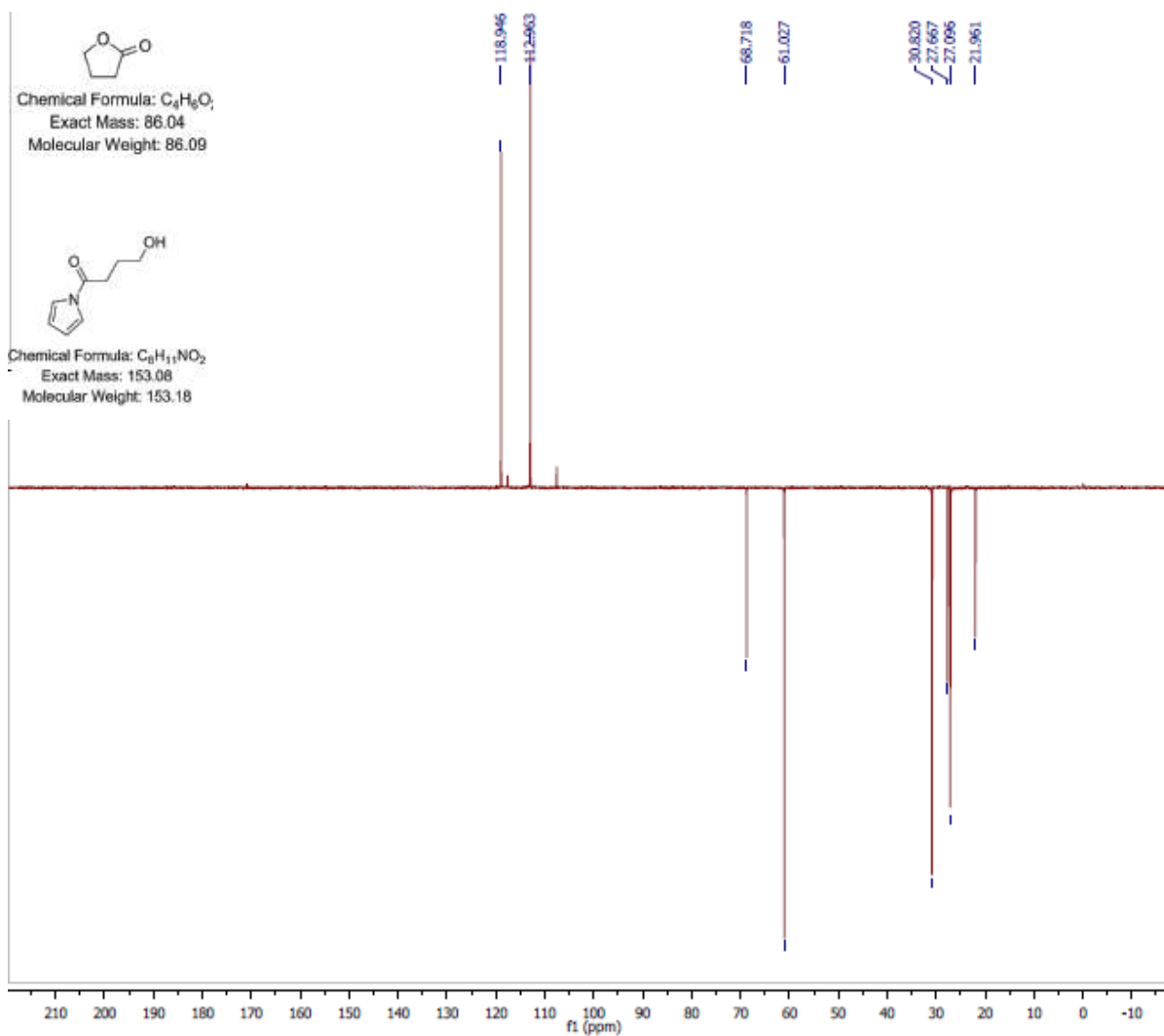
## **LIST OF APPENDICES**

## APPENDIX A

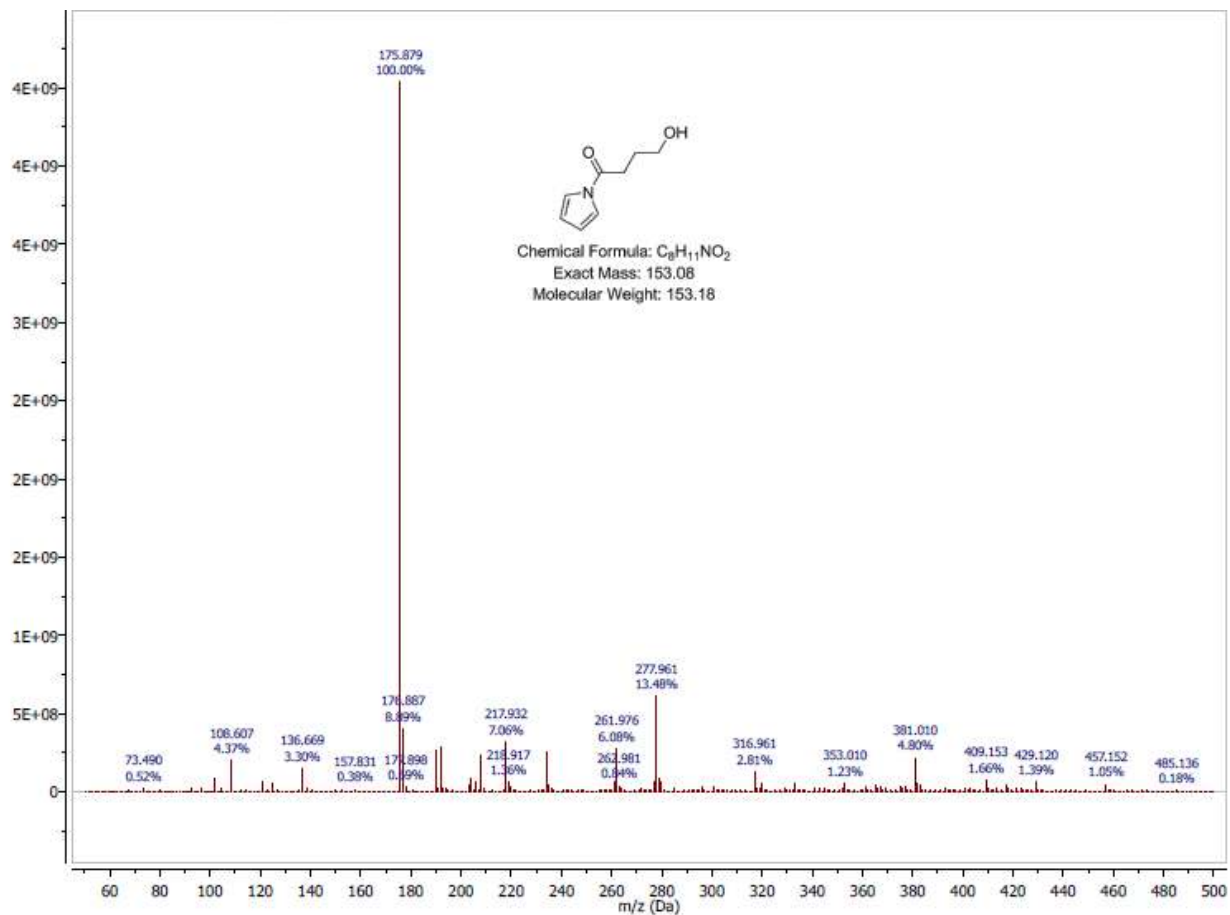
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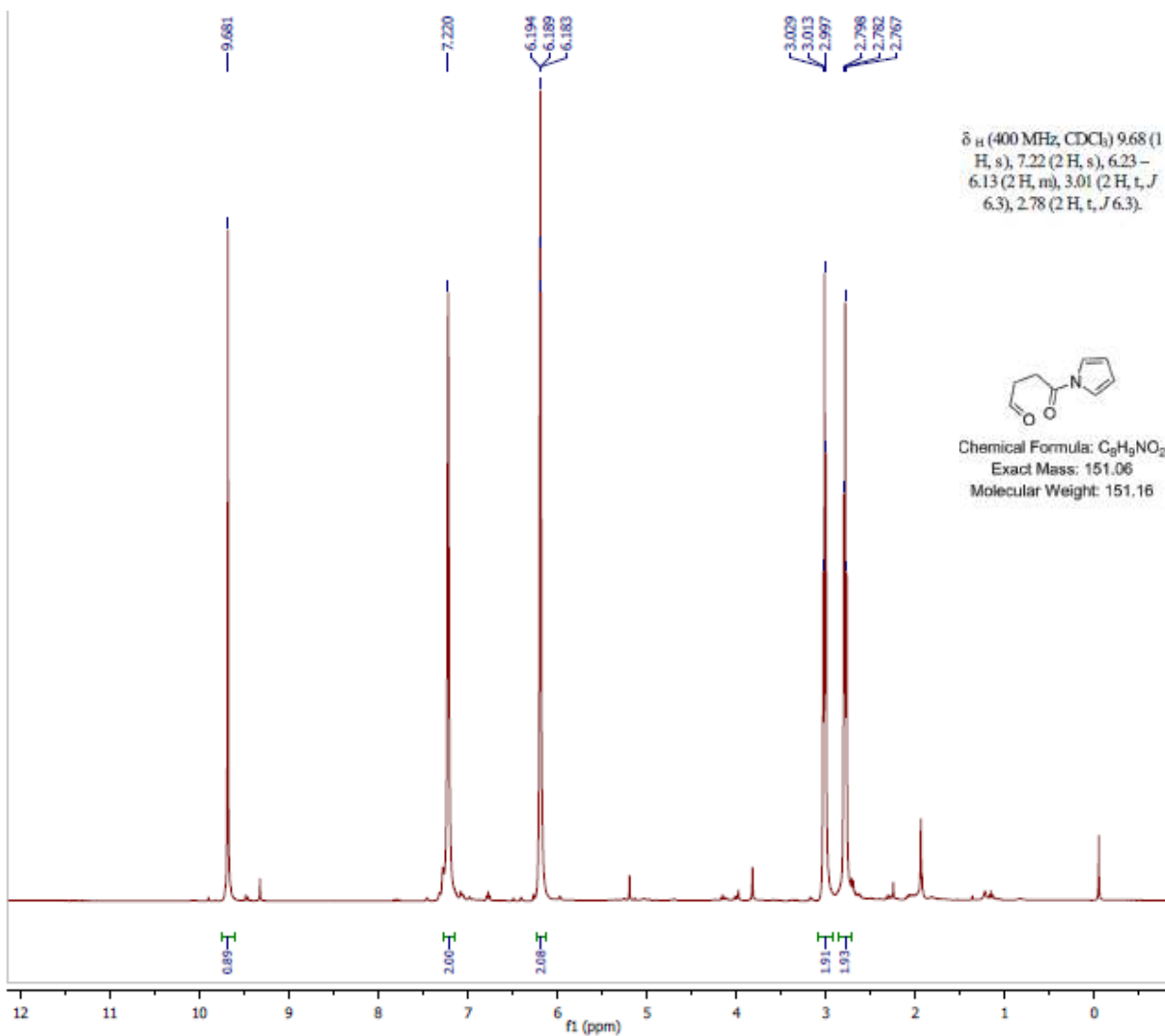


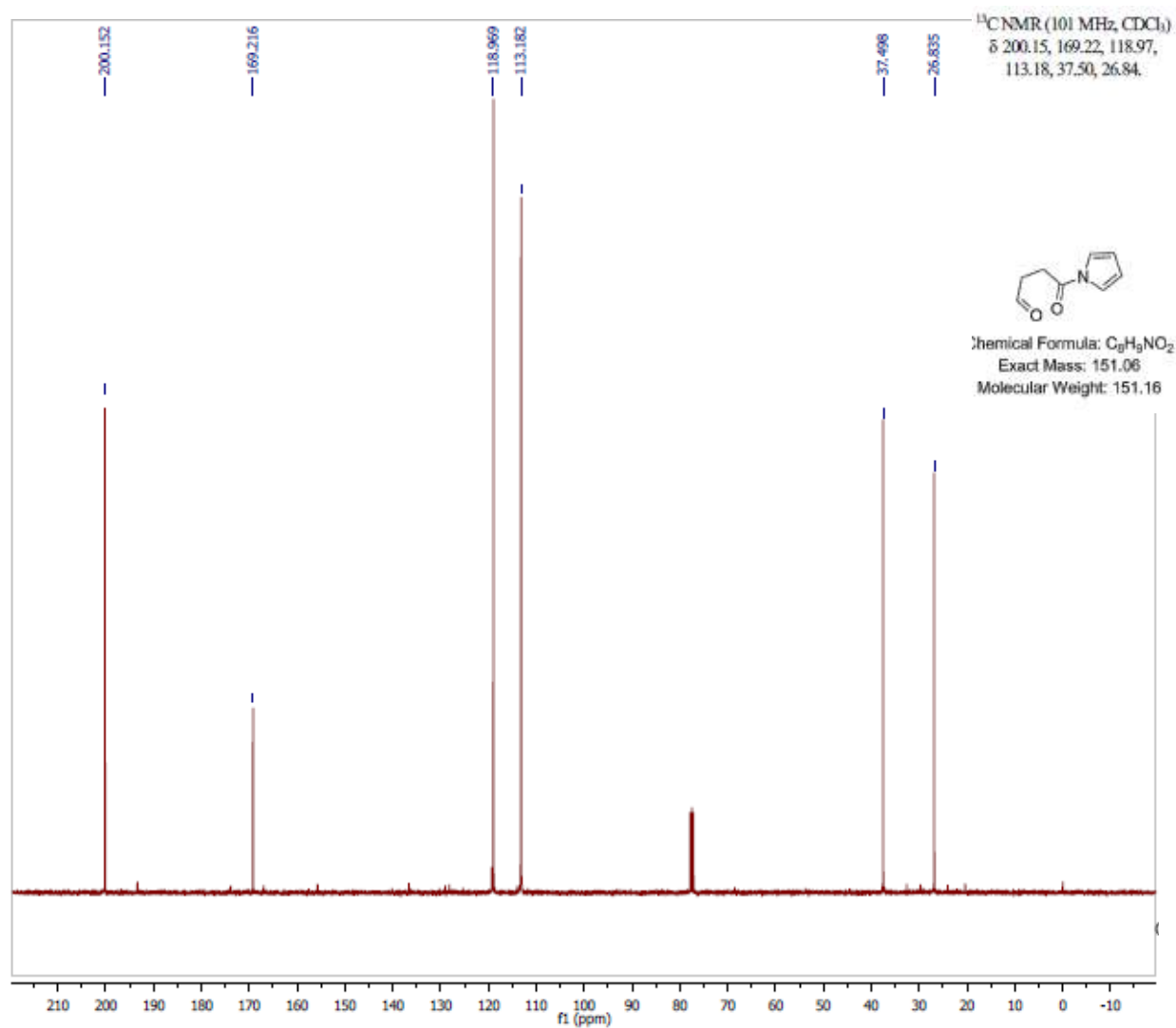


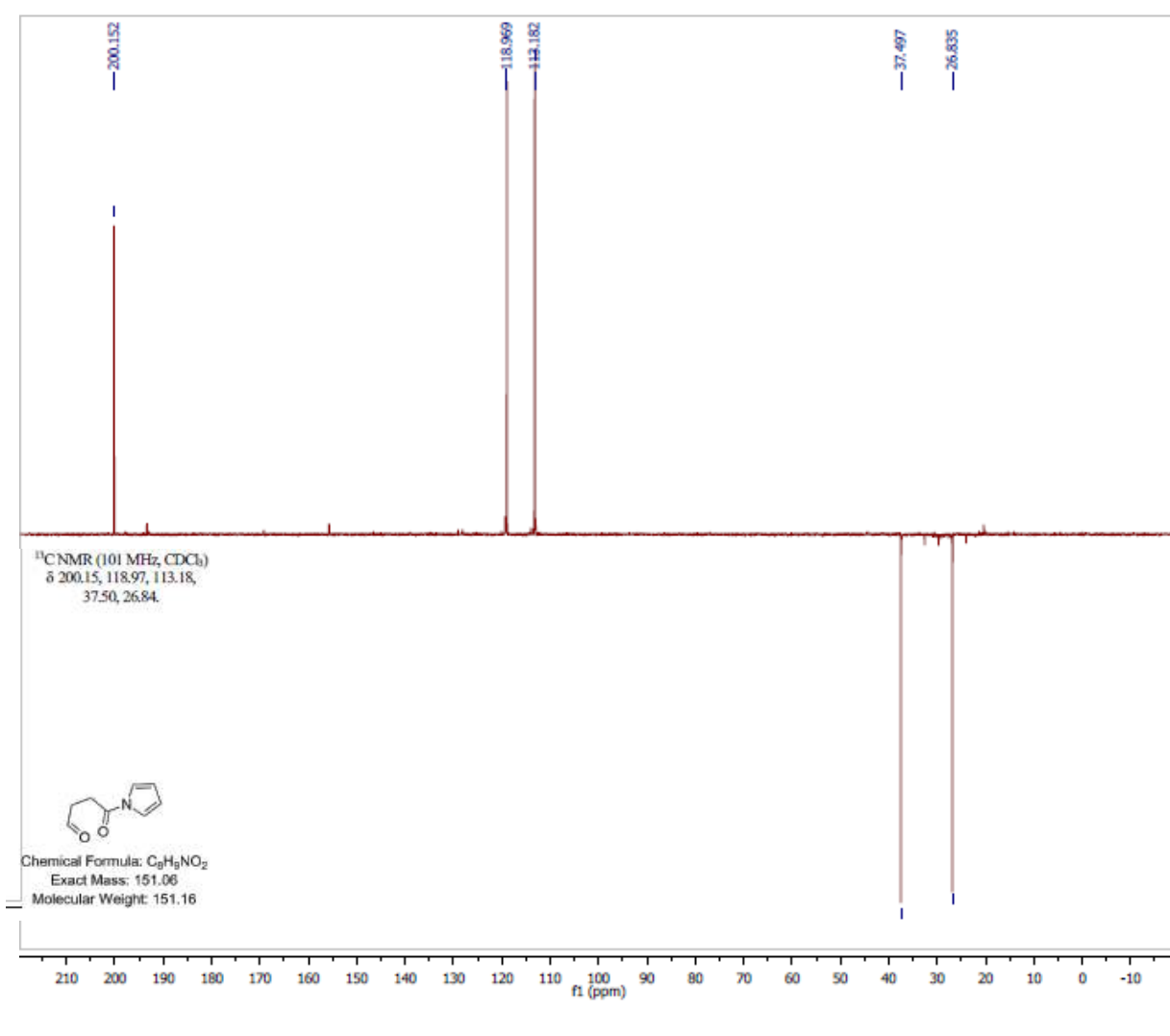


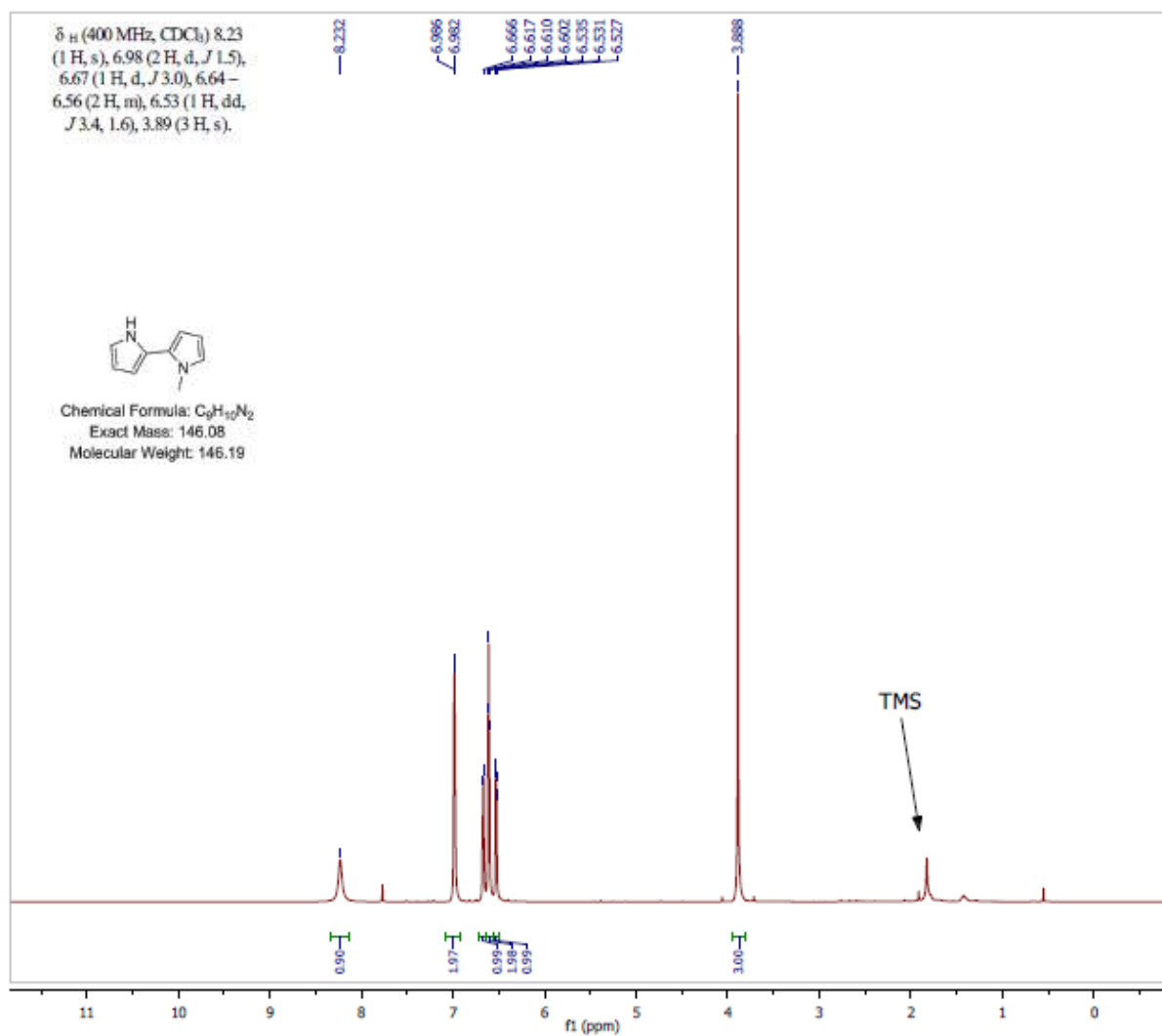


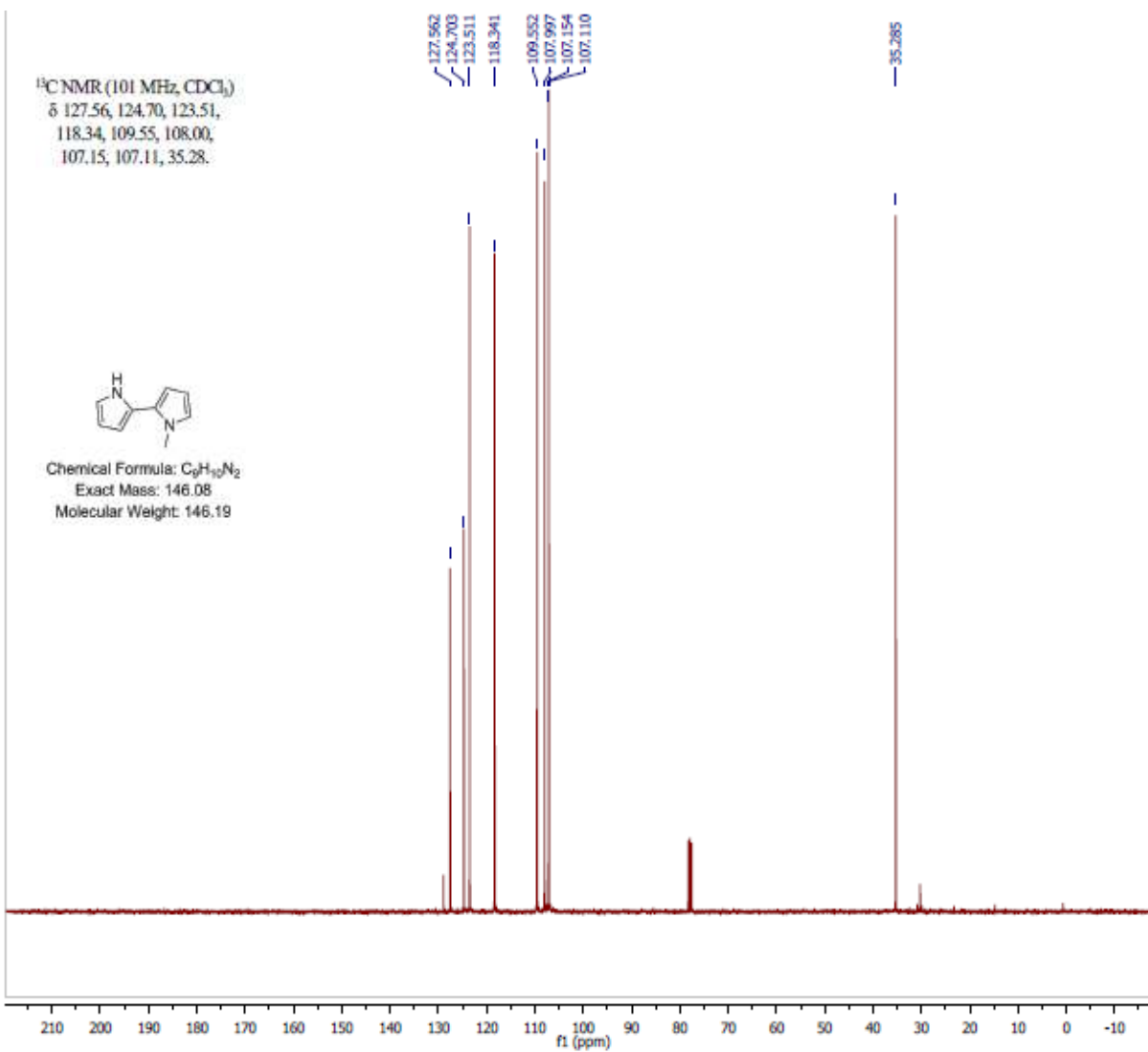


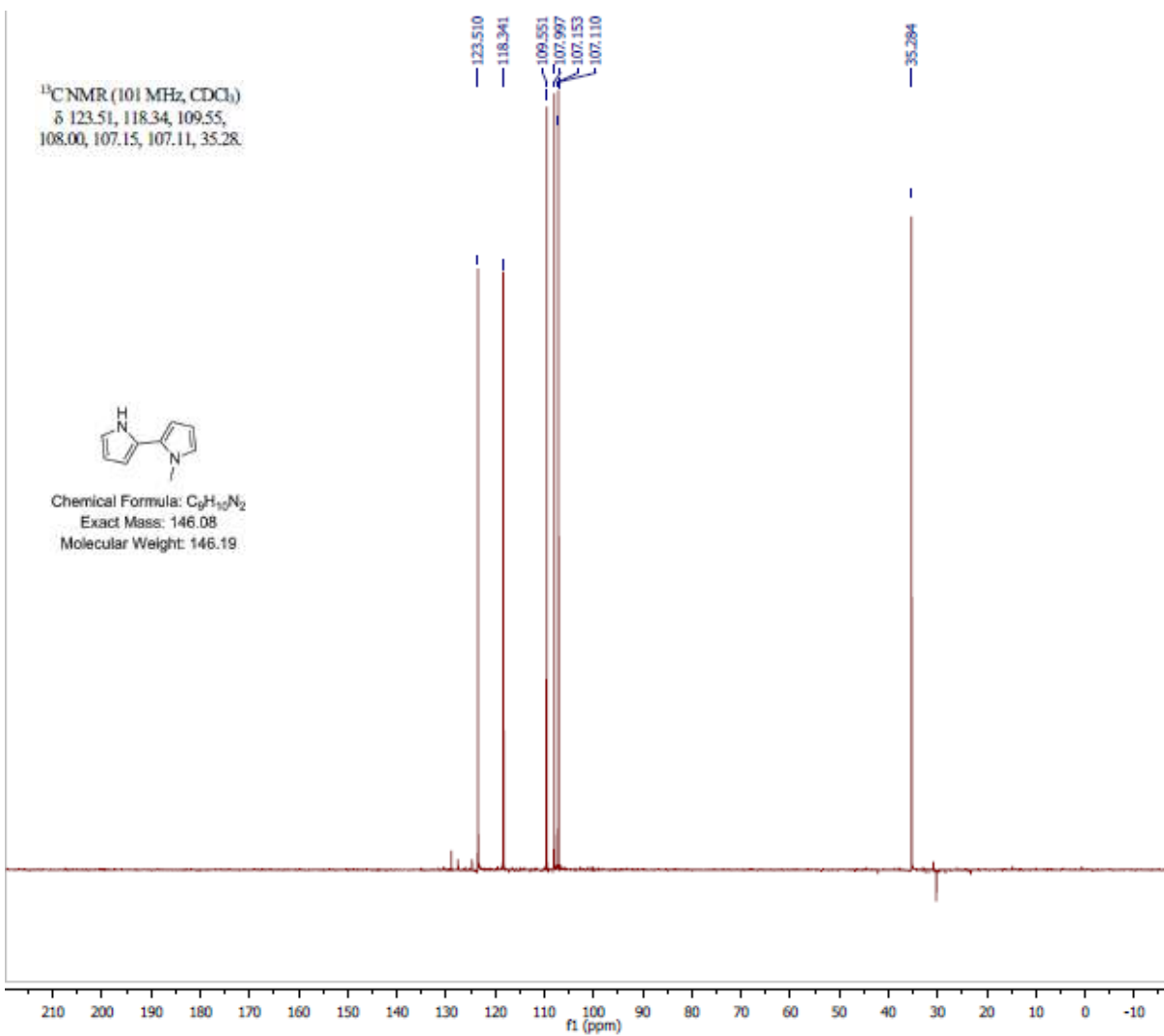


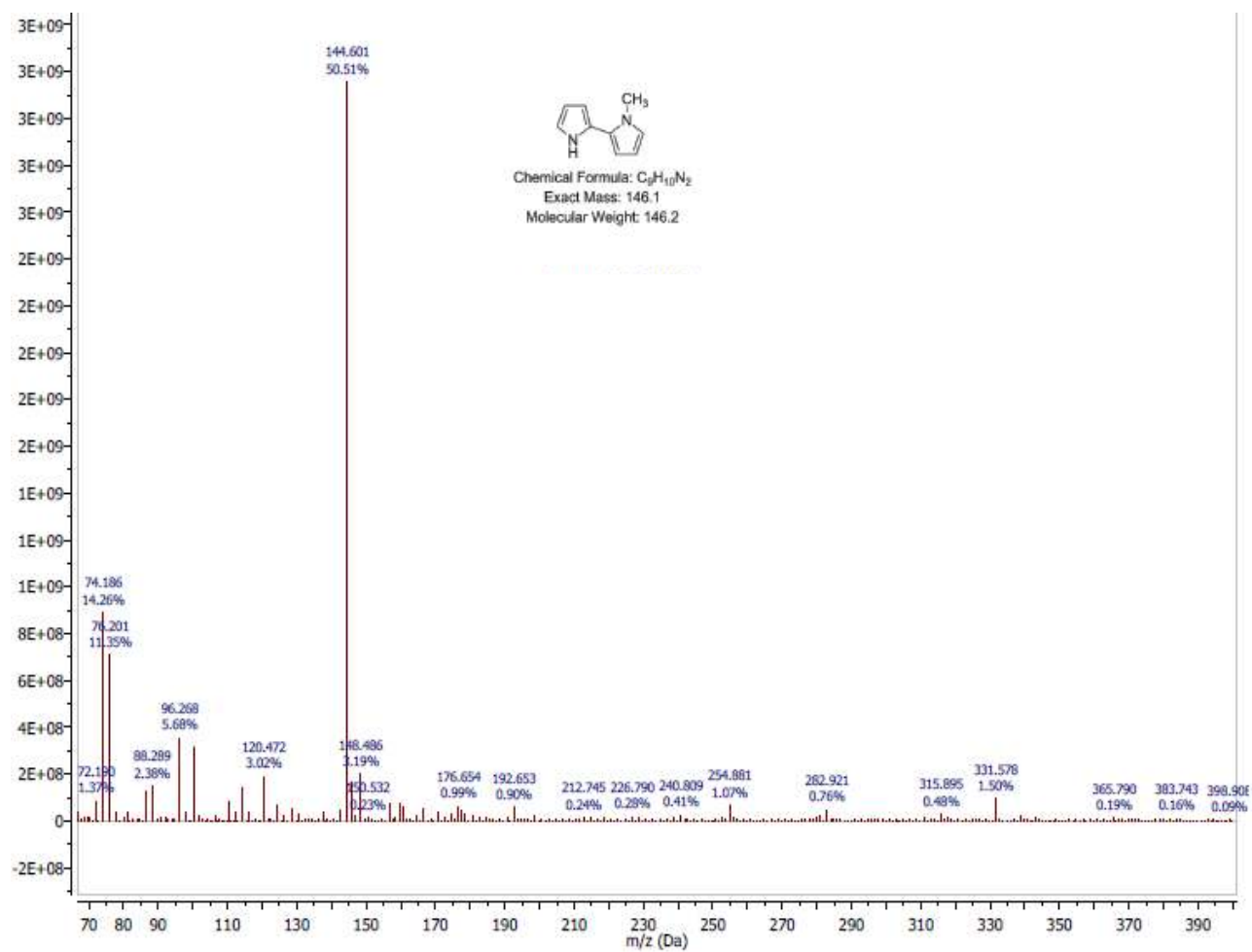




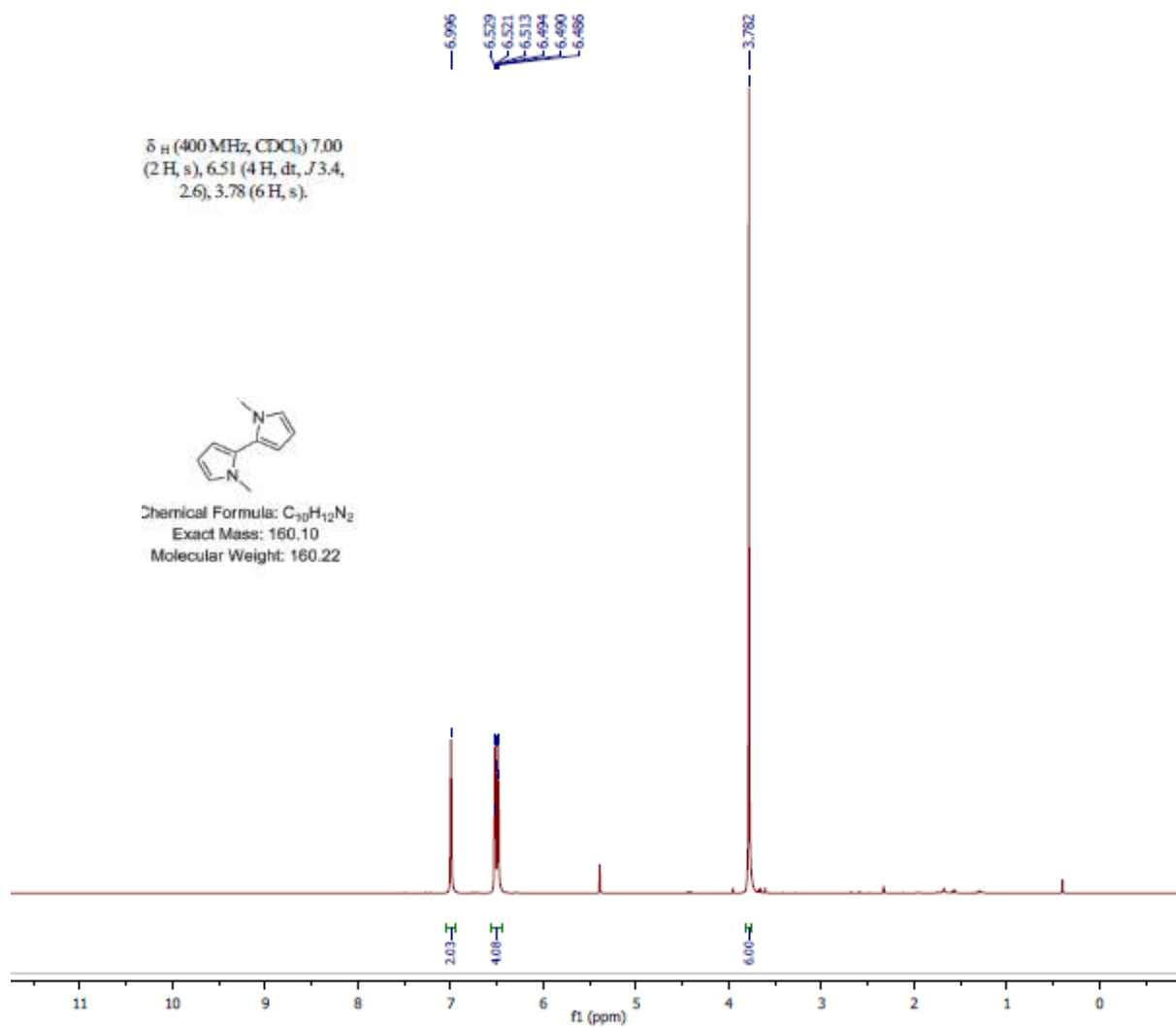


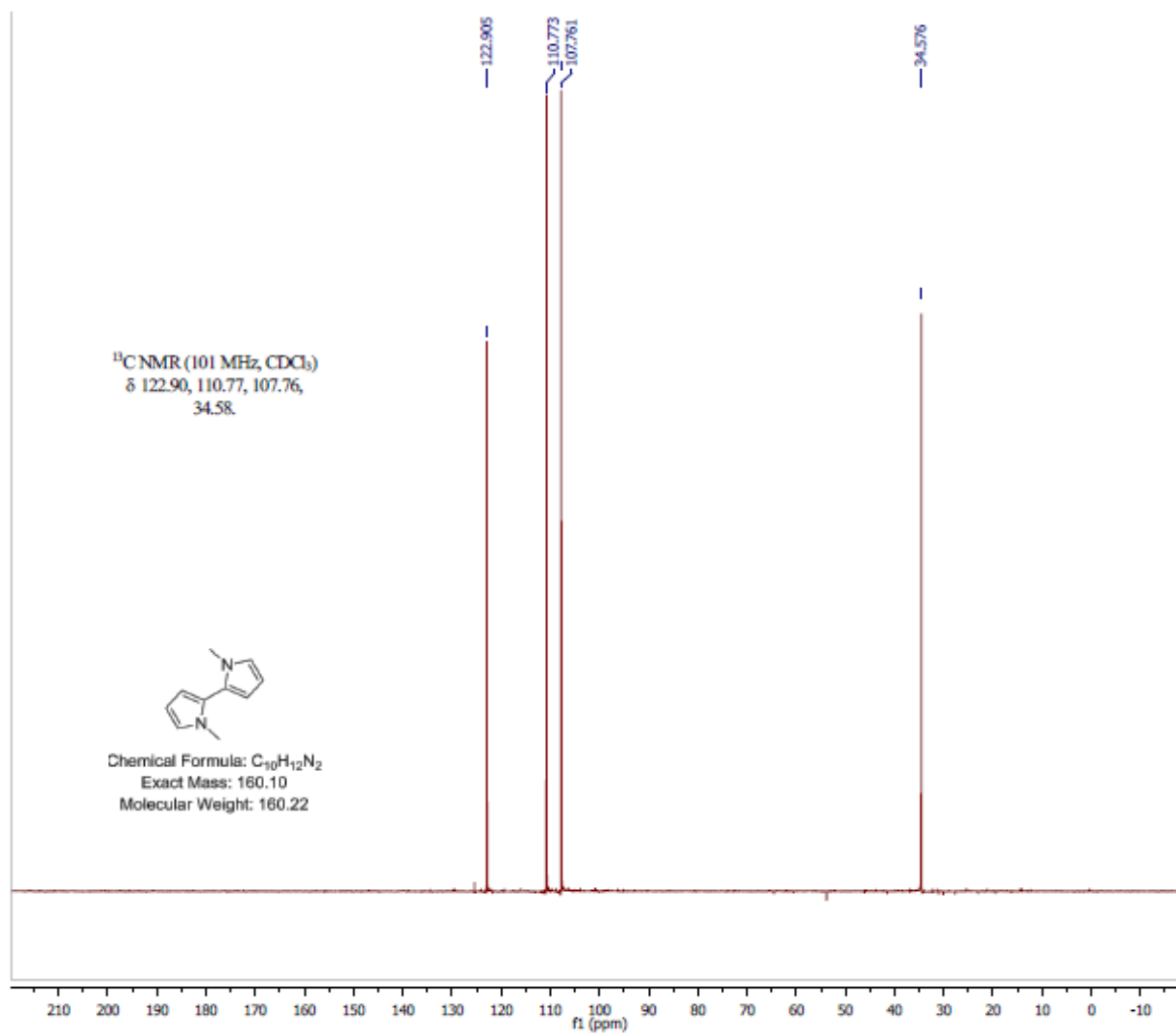


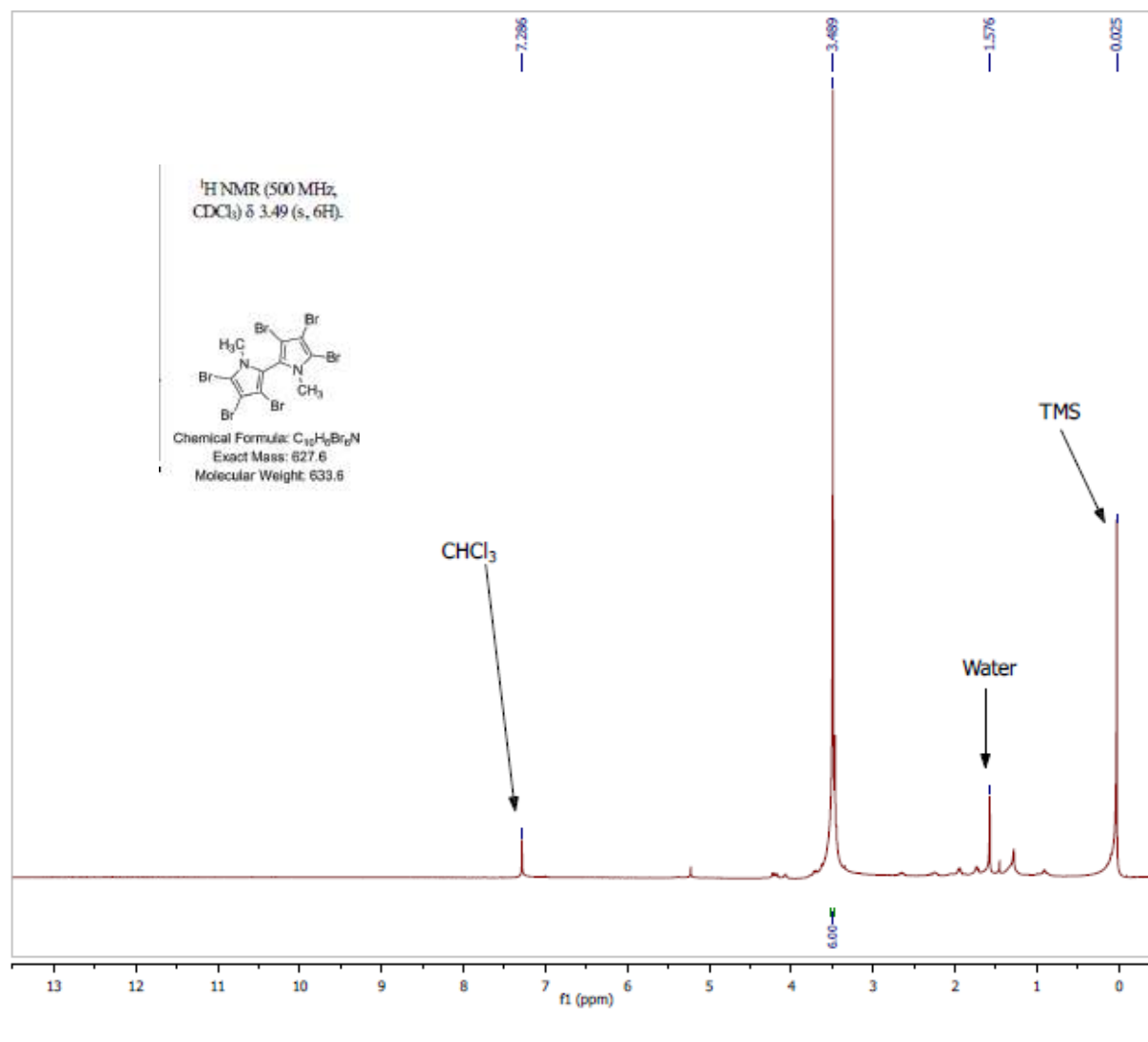


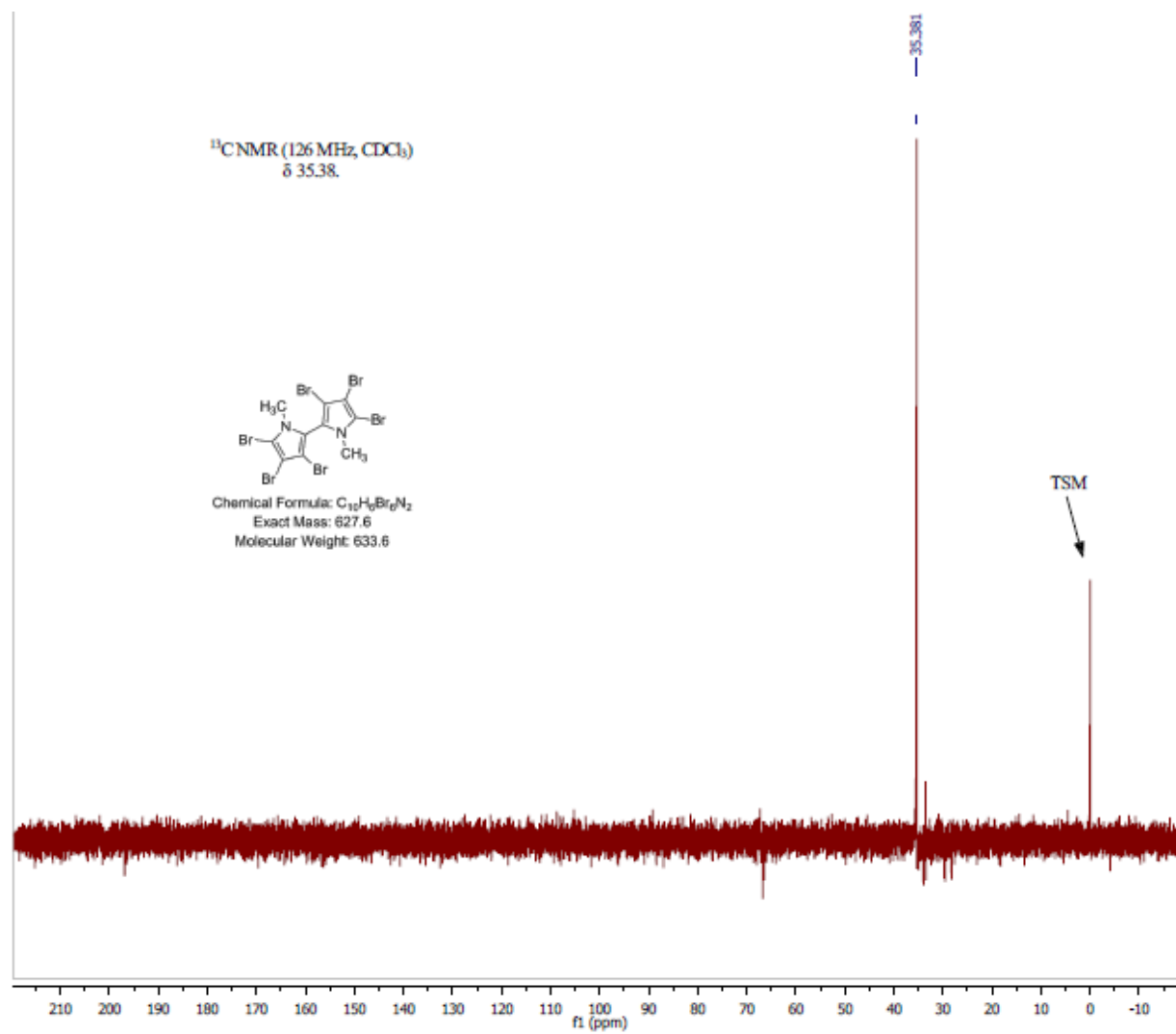


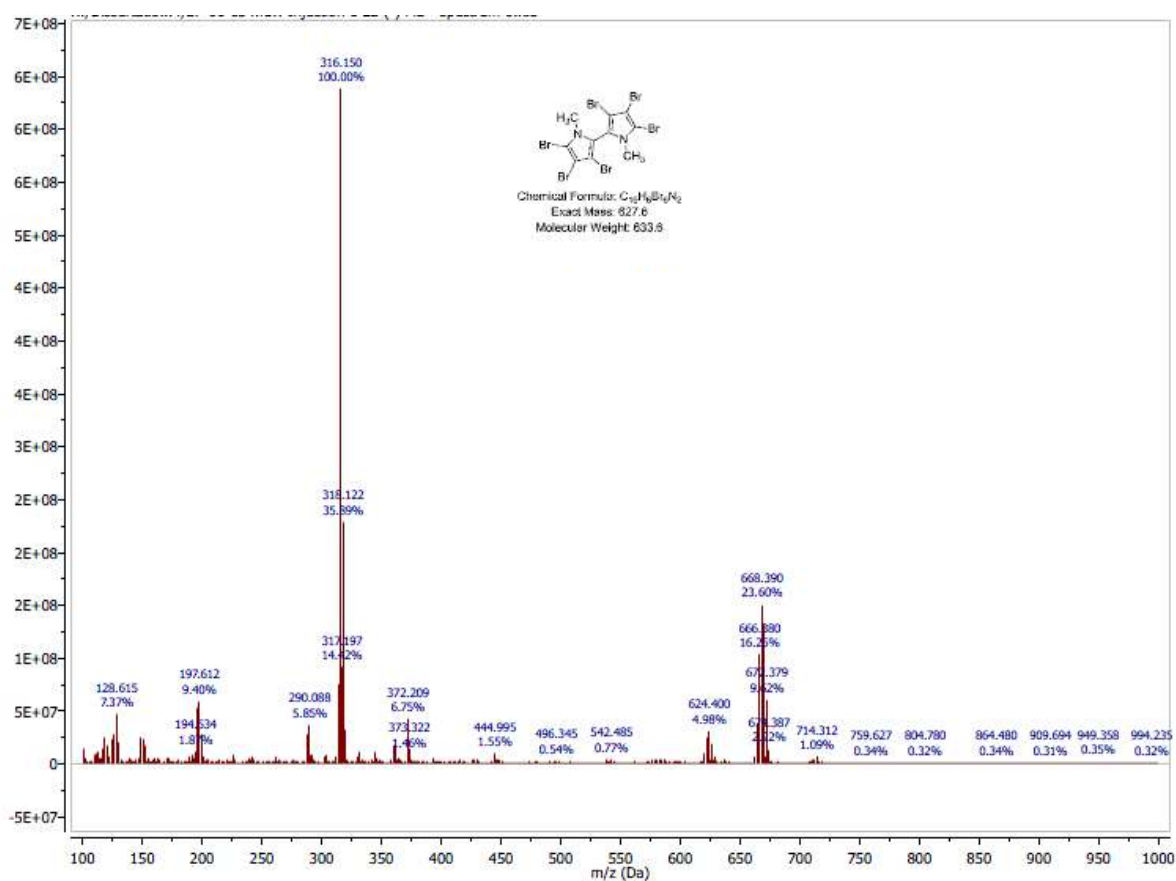


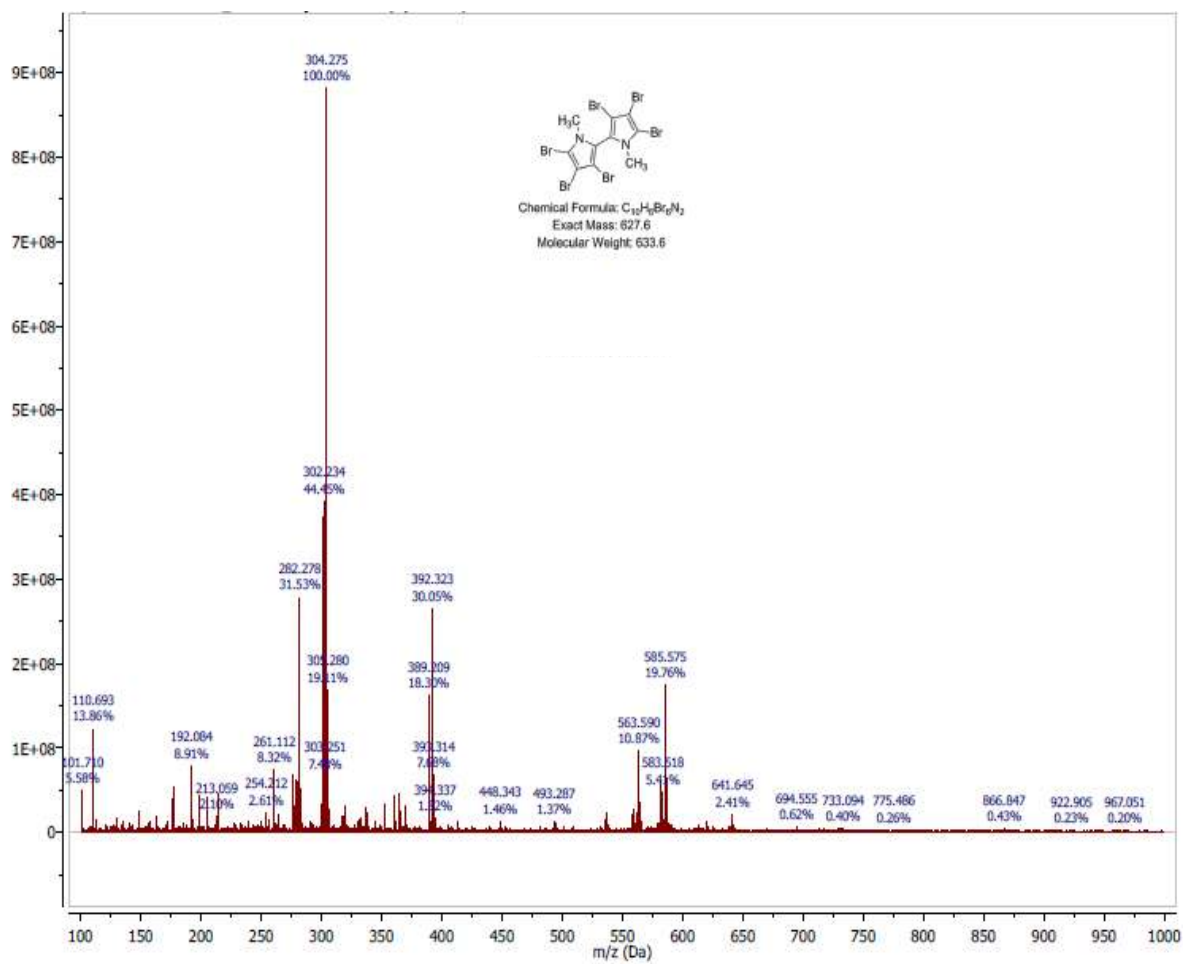






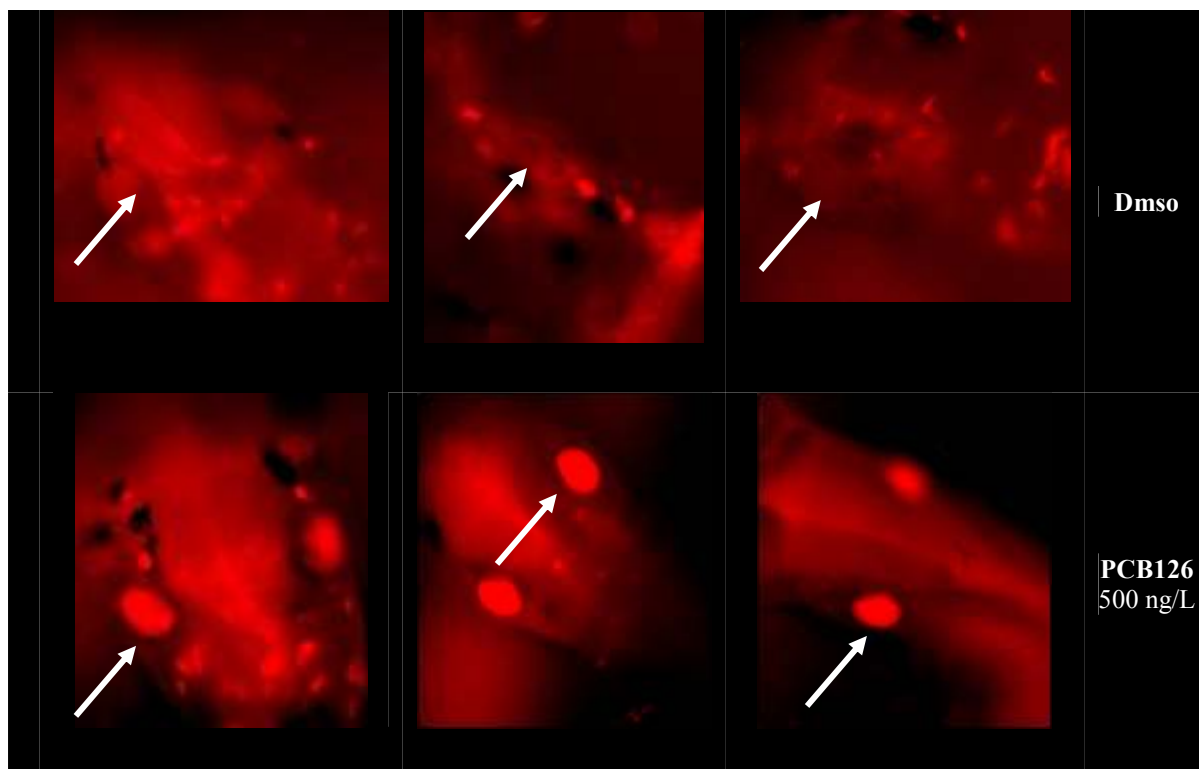




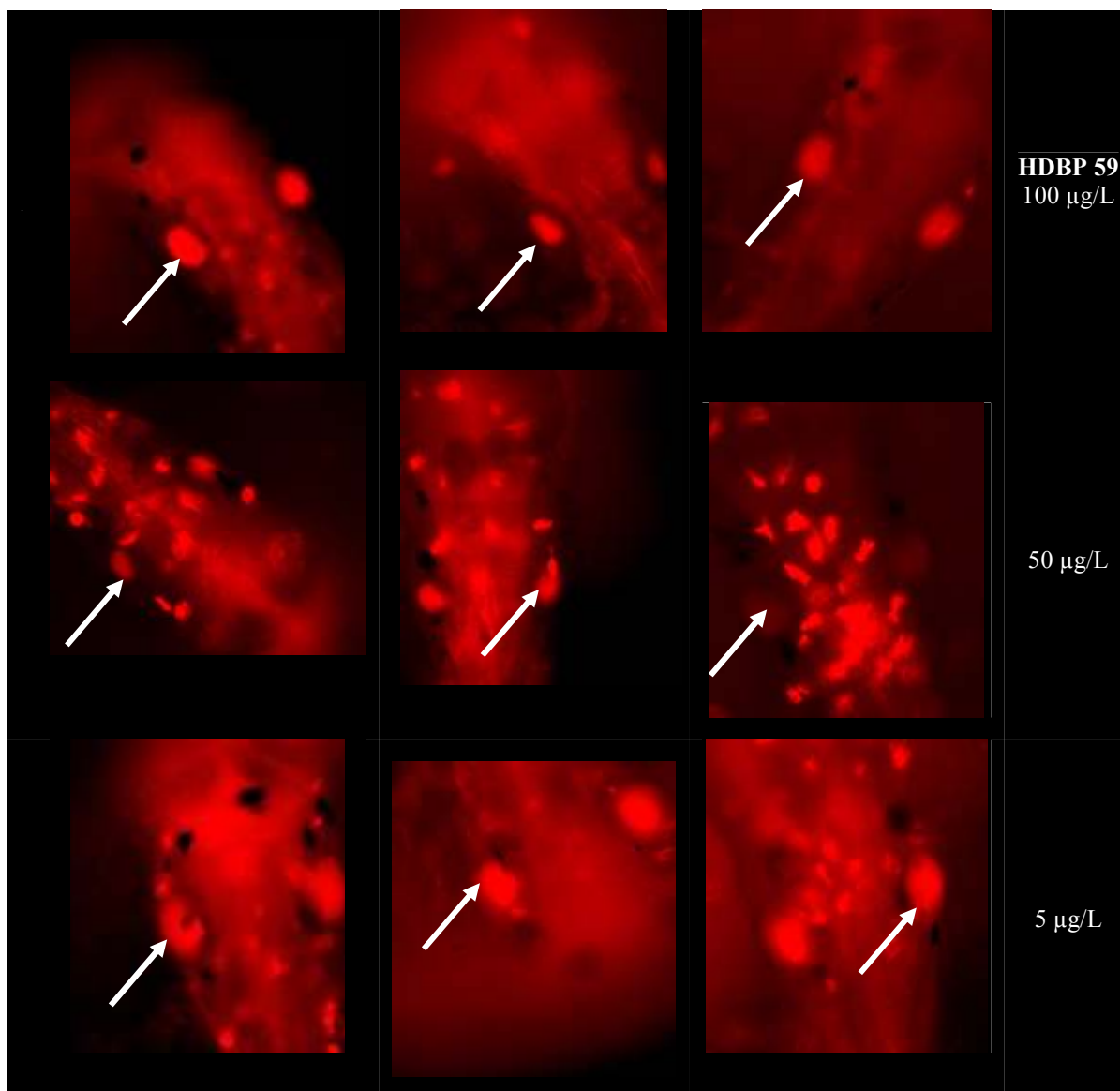


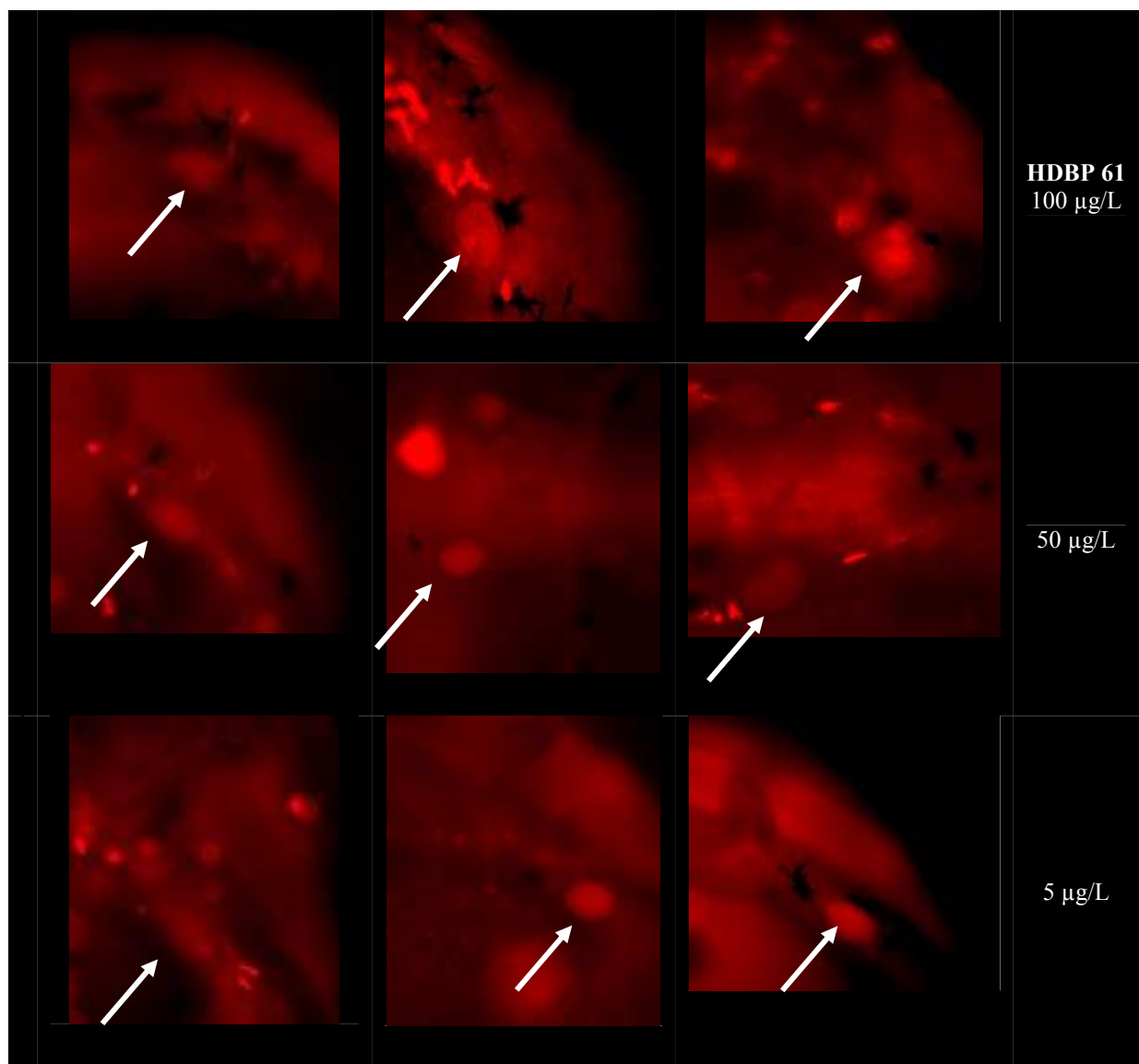
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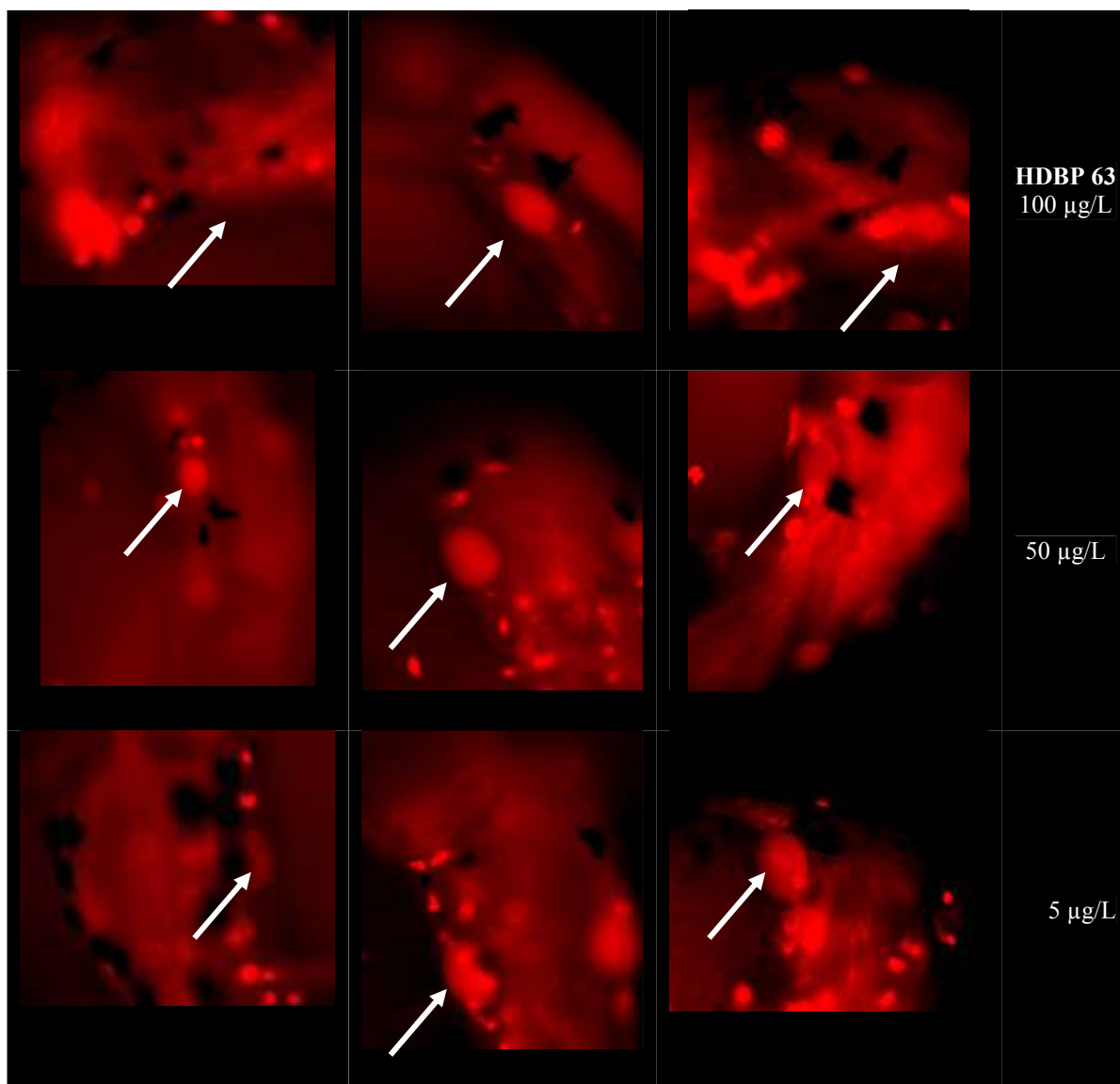
### DIGITAL OF IMAGES OF EROD ASSAY











## **VITA**

Kimberly Sheree Foster was born in Natchez, MS, USA on October 2<sup>nd</sup>, 1987. She received her Bachelor of Science degree in Forensic Chemistry from the University of Southern Mississippi in May 2010, with the distinction of honors graduate. She joined the Master's program in the Environmental Toxicology Research Program at the University of Mississippi in the fall of 2010 and successfully completed all the requirements for a Master's degree in the fall of 2012.